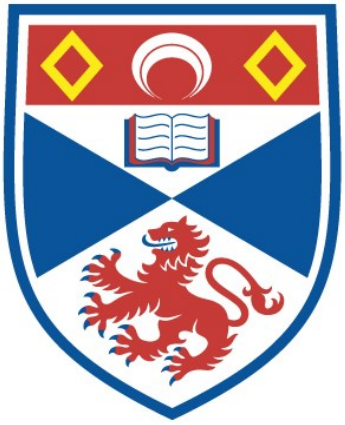
GENETIC CONTROL OF THE EXPRESSION OF SURFACE ANTIGENS IN PARAMECIUM

Catharine McTavish

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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GENETIC CONTROL OF THE EXPRESSION OF SURFACE ANTIGENS IN *PARAMECIUM*

by

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A Thesis submitted for the Degree of Doctor of Philosophy

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DECLARATION

I hereby declare that this thesis is my own composition, and that the experimental work was performed by me alone. Some of the material included in Chapter 2 and 3 has been published jointly with Dr. J. Sommerville in Chromosoma (1980) vol. 78. pp. 147-164. None of the material in this thesis has been submitted for any other degree.

16/8/80

CATHARINE McTAVISH

CERTIFICATE

I certify that Ms Catharine McTavish has spent 24 terms at research work on the genetic control of the expression of surface antigens in Paramecium.

16th August, 1980

SUMMARY

The serotype transformation system of the ciliate Paramecium primaurelia has been examined in order to elucidate the mechanism which controls its switch in gene expression. During temperature induced transformation of stock 168, the cell switches from the synthesis of the G surface antigen protein to that of the D surface antigen protein.

In Chapter I the growth conditions and serotype expression of stock 168 of P. primaurelia have been examined. In Chapter II the organisation of the macronuclear genome has been investigated. The genome of Paramecium is simple, having little repetitive DNA and a complexity only 19 times higher than that of E. coli.

The transcription of the macronuclear genome has been examined in Chapter III. Paramecium, like higher eucaryotes, contains polyadenylated RNA which has a heterogenous size range. This polyA$^+$ RNA is present in the cell at a broad range of intracellular frequencies, the least frequent class of polyA$^+$ RNA being present at approximately 1000 copies per cell, while the higher frequency classes are present at 10^5 - 10^6 copies per cell. The least frequent class of RNA is transcribed from up to 12% of the genome in cells grown at 25°C and expressing the G serotype, while cells grown at 32°C appear to transcribe up to 22% of the genome. PolyA$^+$ RNA has been translated by a wheat-germ in vitro translation system, in which polypeptides of up to 45 Kdaltons were translated.

There appears to be no synthesis of RNA which is larger than polyA$^+$ RNA, in contrast to the situation in higher organisms, although an examination of transcription units showed long nascent RNP.

RNA and protein synthesis has been examined during the process of transformation. There appeared to be little change in either the pattern of RNA synthesis or the spectrum of proteins present. Polysomes containing antigen mRNA were specifically precipitated with antiserum. Although there was some non-specific precipitation, there was an enrichment of RNA in the size range 10 - 12S, the size expected of RNA coding for one antigen subunit.

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INTRODUCTION

The ciliated protozoa are considered to be amongst the simplest of the eucaryotes. They are, however, an extremely successful group, occupying a wide variety of habitats and displaying many different forms. Although they are merely single-celled organisms, the ciliates display an impressive complexity of structural organisation and behaviour.

In spite of the wide variety of forms, the ciliates can be grouped together as a class on the basis of the possession of a number of features (reviewed by Jones, 1974), the most obvious being the large numbers of cilia and the presence of two types of nuclei. In this latter feature the ciliates are unique in the animal kingdom. Some groups of animals possess more than one nucleus per cell, but only in the ciliates are such structurally and functionally distinct nuclei found.

The two nuclei can be distinguished on the basis of size and, because of this, have been termed macronuclei and micronuclei. Functionally too they are quite different, displaying a "division of labour" in the cell. Transcription of RNA is largely or entirely restricted to the large polyploid macronucleus, while genetic events, e.g. meiosis and recombination, are only found in the small, permanently-condensed, micronucleus. In spite of these gross differences, the two nuclei have a common origin, the macronucleus developing from a division product of the micronucleus during the life-cycle. It is therefore analogous to the nuclei of somatic cells and for this reason is often termed the somatic nucleus.

The structure, development and evolution of the ciliate macronucleus is reviewed by Raikov, (1976).

Such a unique developmental process, and the ultimate segregation of nuclear functions, prompts one to ask questions about the nature of the genetic material in the two ciliate nuclei. Is the information content of the macronuclei and micronuclei the same, and is it similarly organised?

It is becoming increasingly apparent that in many eucaryotes, gene expression may be modified as a consequence of genetic reorganisation, at least in somatic cells. For example deletion and splicing of genomic DNA sequences occur in the formation of immunoglobulin genes (Brack et al. 1978., Davis et al., 1980., Weigert et al., 1980). Amplification of certain genes may occur at specific developmental stages, for instance ribosomal gene amplification in oocytes (Tobler, 1975), or as a response to an environmental stimulus, for example the amplification of the dihydrofolate reductase gene in methotrexate treated cells (Alt et al., 1978., Schimke et al., 1978). Insertion elements, similar to those of bacteria, are found in yeast and Drosophila and can insert at various sites in the DNA, changing the linear organisation of the genome and modifying gene expression (Cameron et al., 1979., Potter et al., 1979., Strobel et al., 1979).

In the ciliates there is clearly scope for this type of influence on gene expression, particularly during the development of the macronucleus. There is evidence that some form of DNA reorganisation takes place during macronuclear development

and that, in at least one group of ciliates, the hypotrichs, this is extensive (Ammermann et al., 1974., Lauth et al., 1976).

Probably more general than the modification of gene expression in somatic cells by alterations of the DNA, is regulation of gene expression at the level of transcription and translation. The structural organisation of chromatin may be an important influence on transcriptional activity. This is largely determined by the interaction of histones and non-histone proteins with the DNA (Elgin and Weintraub, 1975). The ciliates possess histones which are similar to those of higher eucaryotes. (Isaacks and Santos, 1973, Lipps et al., 1974, Johnman and Gorovsky, 1976.). Consequently one might expect genetic activity to be similarly influenced in the ciliates.

In higher eucaryotes, messenger RNA appears to be transcribed as a high molecular weight (hnRNA) precursor which is later processed. Such processing involves many different steps including the addition of a methylated 5' capping sequence, specific endonucleolytic cleavage, addition of a polyadenylated sequence, methylation and ligation of cleaved fragments. Selective processing is clearly a potential form of control of gene expression (see Darnell, 1976; 1979). Indeed it has been shown by the work of Early et al., (1980) that two different mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways.

The prevalence of repetitive DNA, both in the eucaryotic

genome and in hnRNA transcripts, is another feature which has been commonly implicated in gene control. For instance this observation has prompted Davidson and co-authors to propose a quite different function for hnRNA, which they suggest acts as a regulatory transcript for the co-ordinate control of structural genes (Davidson et al., 1977). It is suggested that the repetitive transcripts of the hnRNA modify structural gene transcription by interacting with complementary repetitive sequences in the DNA, or alternatively influence RNA processing by interacting with complementary hnRNA molecules. (Scheller et al., 1978).

In ciliates the evidence to date suggests that not only is there very little repetitive DNA in the macronucleus (Ammermann et al, 1974., Yao and Gorovsky, 1974 Cummings, 1975., Lauth et al., 1976., Pelvat and de Haller, 1976) but there is also very little if any hnRNA (Prescott et al., 1971). Gene control systems involving either one or both of these elements may be lacking or rudimentary in the ciliates.

Control of gene expression is clearly very complex in the eucaryotes and probably acts at a number of levels. The difficulty in analysing control processes is partly due to the organisational complexity of the higher eucaryote. Bacteria, on the other hand, have quite simply organised genomes and the function of much of the DNA has been determined. In addition, the control of various bacterial genes has been elucidated, specifically as the Jacob-Monod regulatory mechanism (reviewed by Lewin, 1974).

However genetic control mechanisms in eucaryotes appear to be fundamentally different from those of bacteria (in spite of the similarity in the sequence of bacterial and putative eucaryotic promotor regions (e.g. Gannon et al., 1979)). It seems likely that useful information on eucaryotic gene control mechanisms might be derived from studies on primitive eucaryotes. The ciliates are a suitable group of organisms to study for a number of reasons. Firstly, the ciliate genome is much less complex than that of higher eucaryotes. Secondly, they can be cultured and cloned very easily. Thirdly, if it is generally true that the ciliates possess very little repetitive DNA and hnRNA, then this excludes at least some of the apparant complexity of higher eucaryotic gene control.

In the ciliates an excellent system to study the question of genetic control mechanisms is that of the serotype transformation system in Paramecium. This has been examined in great detail by a number of authors since Rössle, in 1905 observed that paramecia could be immobilised by placing them in a dilute solution of homologous antiserum.

It has since been established that this phenomenon is due to the presence on the cell surface of a particular protein, termed variously the antigen protein, the serotype protein or the immobilisation antigen. Antiserum prepared against paramecia contains antibodies to this protein and the effect of homologous antiserum is to agglutinate the cilia and thus immobilise the cells.

Apparantly resistant cells are found to have replaced this protein with another similar, but antigenically different,

protein. In all, up to twelve different cell types (or serotypes) can be found, each having a diffent antigen protein on the surface (reviewed by Beale, 1954). Genetic analysis has shown that each antigen is the product of a different genetic locus (reviewed by Preer, 1968) but only one is expressed under a particular set of conditions and, in heterozygous cells, only one allele is expressed. These proteins therefore display both intergenic and interallelic exclusion (reviewed by Finger, 1974).

The antigen protein itself is very large (310,000 daltons) and there in some disagreement about whether it is composed of subunits. The biochemical work of Steers (1965) suggests that it is composed of 3 identical subunits, each made up of 3 different subunits, and genetic evidence supports this subunit hypothesis (Finger et al., 1966). Nevertheless some authors claim that the antigen protein is a single globular polypeptide (Reisner et al., 1969, Hansma, 1975.).

The synthesis of the antigen proteins has been examined (reviewed by Sommerville, 1970) but very little is known about control of their synthesis.

A number of questions can be asked about the control of the expression of antigen genes. For example, is the control at the level of transcription or translation? If the antigen is composed of subunits, are they transcribed as a polycistronic mRNA or as separate RNA molecules? How many copies of the antigen gene are there in the macronucleus and are they all transcribed? By what mechanism does a change in environmental conditions cause a switch in gene expression? How are the phenomena of intergenic and interallelic exclusion mediated

at the molecular level?

Some of these questions may be difficult to answer, since little is known about gene organisation and transcription in Paramecium and other ciliates compared to the extensive information available on higher eucaryotes. An attempt has been made, therefore, to examine some general aspects of DNA organisation and transcription in Paramecium, before going on to consider the control of expression of the antigen genes.

In Chapter I, the growth conditions and serotype expression in stock 168 of P. primaurelia are examined. In Chapter II the organisation of the macronuclear genome is investigated and in Chapter III the characteristics of RNA transcription are described. In Chapter IV the process of serotype transformation is examined with respect to the observations in the previous chapters and a possible mechanism of control of the antigen genes is considered in the general discussion.

CHAPTER I

Growth and Maintainance of Paramecium

1. Introduction

Paramecium aurelia is found in fresh-water environments all over the world. Many stocks of P. aurelia have been isolated, but they fall into only fourteen distinct species (Sonneborn, 1975).

P. aurelia can easily be cultivated in the laboratory if provided with a vegetable infusion to act as a food source for the micro-organisms on which the paramecium feed. Various vegetable infusions have been used successfully to cultivate paramecia, the most widely used being Cerophyll infusion, grass infusion, lettuce infusion and Vegemite. Micro-organisms grown on these infusions include bacteria, yeast and algae. However, the most commonly used food organism is Klebsiella aerogenes. (Culturing techniques are reviewed by Sonneborn (1970)).

The presence of bacteria in cultures of Paramecium is inconvenient for some purposes since it complicates the interpretation of metabolite-uptake experiments. The use of axenic cultures of paramecia for such experiments would be preferable. Paramecia have been grown axenically since 1948 (Van Wagtendonk and Hackett, 1949). However, the nutritional requirements of paramecia are complex and the currently-used axenic medium is only semi-defined (Van Wagtendonk and Soldo,

1970). Furthermore, the growth rate in this medium varies between stocks and species, some growing extremely well and others less so. For example, many stocks of P. tetraurelia grow well in axenic medium whereas stocks of P. primaurelia do not grow so well and for most purposes are routinely grown in monoxenic culture.

When grown in bacterised grass medium at 25°C, many stocks of P. primaurelia express the G serotype (Beale, 1952). At higher temperatures, for example 32°C, the D serotype is expressed. A culture of paramecia expressing the G serotype can be "transformed" to expression of the D serotype by increasing the temperature to 32°C. The switch from one serotype to the other occurs rapidly, the new serotype being detectable, by immobilisation, one to two fissions after the temperature increase. The transformation process will be discussed in more detail in Chapter IV.

The serotype of a particular culture of cells is ascertained by its reaction to antisera. Cells of the G serotype are immobilised by antisera prepared against homologous G cells. Cells of the D serotype are immobilised by antisera prepared against homologous D cells.

Antiserum is prepared by injection, into a rabbit, of a homogenate of cells or, more routinely, by injection of a ciliary extract of the cells. The antigen protein may be purified from the ciliary extract and a solution of the protein injected for antiserum production.

2. Materials and Methods

(i) Stocks

Paramecium primaurelia stock 168 was routinely used. This was obtained from the cultures of Professor G.H. Beale, Institute of Animal Genetics, University of Edinburgh.

(ii) Culturing

(a) Bacterised grass culture

Concentrated grass medium:- 100g of grass pellets from Standingstone, Midlothian was added to 1 litre of distilled water, boiled for 15 minutes and filtered through 4 layers of muslin. The concentrated medium was sterilised by autoclaving at 15 lb. per sq. in. for 15 minutes.

Diluted grass medium:- 50 ml of concentrated grass medium was diluted to one litre and made up to 1mM Na_2HPO_4. The medium was autoclaved at 15 lb. per sq. in. for 15 minutes.

Maintainance of bacteria:- *Klebsiella aerogenes* was maintained on Oxoid nutrient broth agar slants or plates and was subcultured every 4 weeks.

Bacterised grass medium:- 10 ml of sterile grass medium was innoculated with a loopful of bacteria from a stock plate, or slant, of *K. aerogenes*. This was incubated overnight at 37°C, diluted to 20 ml with distilled water and the pH adjusted to 7.0-7.5 with 0.1M NaOH.

(b) Culturing of large volumes of paramecia

Each litre of culture was grown from a single cell. Routinely one cell from a stock culture maintained at room

temperature in grass medium and subcultured at 2 week intervals, was transferred to 0.5 ml bacterised grass medium in a depression slide. Normally nine such isolates were made at one time. Each depression slide, containing nine depressions was placed in a petri-dish containing damp filter paper and growth continued at either 25°C or 32°C. After 3-4 fissions, the cells were transferred to 2ml bacterised medium. This volume was made up to 10ml, by the addition of sterile grass medium, when the culture had "cleared" i.e. had become clear due to the removal of bacteria by the dividing paramecia. The 10ml tube was later transferred to 100ml sterile grass medium in a 500ml flask and this was subsequently transferred to 1 litre of medium in a 2-5 litre Thompson bottle which was placed horizontally to maximise the surface: volume ratio. Culturing in Thompson bottles was continued until the culture had cleared and cells were in late log phase. Cells were normally harvested at this stage.

(c) Harvesting cultures of paramecia

The culture of paramecia (usually 5-10 litres) was filtered through 4 layers of muslin and one layer of cotton-wool. Cells were harvested from the filtered culture by pelleting in 10ml pear-shaped flasks at 1000rpm for a few seconds in an M.S.E. oil-testing centrifuge. The supernatant was aspirated off, leaving a dense suspension of paramecia in the bottom 5ml. This was transferred to a small beaker.

The process was repeated until all the culture had been harvested. The dense suspension was recentrifuged and the pellet of cells washed at least twice with 100ml of maintainance solution (MS). Maintainance solution was 13mM NaCl, 2mM KCl, 0.14mM $CaCl_2$, 2mM KH_2PO_4, 2mM Na_2HPO_4. After the final wash, the cells were pelleted from MS by spinning at 1000rpm for 5 minutes. This resulted in a packet pellet of cells. The supernatant was removed and the pellet used in further experiments. Usually a small sample was removed from the pellet at this stage to test the serotype of the culture.

(d) Axenic culture

The culture medium used was that described by Van Wagtendonk and Soldo (1970). Cells previously grown in bacterised grass medium were transferred for growth in axenic medium by the following method:-

Individual animals from a monoxenic culture were transferred, by micropipette, to 1 ml of axenic medium in the first depression of a sterile depression slide contained in a sterile petri-dish. Between 20 and 40 cells were transferred into the depression, introducing as little medium as possible with each cell. All cells were then transferred to the second depression containing 1 ml medium and then to a third depression. The cells were left in the third depression for one hour and a second series of three transfers made using a fresh micropipette. In all, 6-7 series of three transfers were carried out, with a gap of one hour between

each series. At the end of this time all bacteria had been removed by dilution and by the feeding activity of the paramecia. All transfers were carried out under a sterile hood and, at the end of the transfers, sterility of the final culture was determined by streaking a sample on to a nutrient broth plate which was incubated at 37°C and examined the next day. A piece of sterile damp filter-paper was placed beneath the depression slide in the petri-dish to reduce evaporation of the axenic medium. When the cells had divided a few times, the culture was transferred aseptically to a test-tube containing 10ml of sterile axenic medium.

(iii) Serotypes

(a) Determination of the serotype of a culture of paramecia

Antisera were initially a gift from Professor Beale. To a small volume of a culture of paramecia containing more than 100 cells (usually 0.1 ml), was added an equal volume of either anti-G or anti-D serum diluted with MS. The dilution varied with the litre of the antiserum, but usually a dilution of 1:100 was adequate. Homologous cells were immobilised within 30 minutes but heterologous cells remained swimming indefinitely. A culture was defined as being of pure serotype if 95%, or more, of the cells were immobilised within 30 minutes. Usually, however, in cultures grown from a single cell, 99-100% of the cells were immobilised by one antiserum.

(b) Preparation of antiserum

A washed pellet of cells of pure serotype, either G or D, was suspended in 4 volumes of salt-alcohol (0.045% NaCl, 15% alcohol). This solution removed the cilia from the cells and solubilised the surface antigen protein. Cells were kept in this solution, with occasional mixing, for 2 hours at 4°C. After this time, cells were removed by centrifuging at 20,000 x g for 5 minutes in a Sorvall refrigerated centrifuge. The supernatant was removed and dialysed against several changes of 0.9% NaCl to remove the alcohol and to make the extract isotonic to rabbit blood.

The extract was injected as a multiple emulsion (Herbert, 1965). Two ml or less of the dialysed extract was injected, using a 1 inch 23g needle, into a mixture of oil and emulsifier (9 parts drakeol : 1 part arlacel). An emulsion was made by repeated passage through the needle. A second emulsion was made by injecting the first emulsion into an equal volume of 2% Tween 80 in 0.9% NaCl. This mixture was passed repeatedly through the needle until a free-flowing solution was obtained. The multiple emulsion was injected subcutaneously into the back of a Dutch rabbit. A booster injection was given 4 weeks after the initial injection. After six weeks from the first injection, 5ml of blood was removed from the marginal ear vien and allowed to clot overnight at 4°C. The clot was removed and any remaining blood cells were centrifuged out of the serum at 1000 x g for a few minutes. The serum was removed and heated at 57°C for

30 minutes to destroy complement. The titre and specificity of a sample of the serum was tested and, if satisfactory, the remaining serum was stored frozen at -80°C. More blood (10-20 ml) was later removed from the rabbit and treated as above. The rabbit was bled occasionally over the next few months to build up stocks of antiserum.

(c) Determination of antiserum titre

A series of dilutions of the antiserum were made using MS. Dilutions were usually 1:2, 1:5, 1:10, 1:50, 1:100, 1:200, 1:500, 1:800, 1:1000, 1:2000 and 1:5000. There was a control consisting of MS with no added antiserum. A few drops of homologous pure serotype culture containing 100 or more cells, were added to each of 12 depressions in depression slides. An equal volume of the 11 dilutions of antiserum and the control were added to the 12 depressions. The time taken for each dilution to immobilise the cells was measured. The results were graphed on logarithmic paper and the dilution at which all cells were immobilised in 2 hours determined by interpolation. This number is defined as the titre value. Each antiserum was tested for cross-reactions with the other serotype by adding some antiserum to heterologous cells at a dilution 10 times less than that required to immobilise homologous cells in 30 minutes. Any antiserum causing immobilisation in these circumstances was rejected.

(d) Double diffusion

An alternative method of detecting cross-reaction and for comparing the antiserum with another was that of double-

diffusion. 20 ml of 1% agar in 0.9% NaCl was poured into a sterile petri-dish and allowed to set. Wells were punched 3mm apart in a hexagonal pattern around a central well. To compare various batches of antisera, 20 µl of homologous salt/alcohol dialysate was pipetted into the central well and 20 µl of the various antisera pipetted into the surrounding six wells. To test cross-reactions of an antiserum with the alternative serotype, 20 µl of heterotogous salt/alcohol dialysate was pipetted into the central well and 20 µl samples of antisera to be tested pipetted into the surrounding wells. Diffusion plates were incubated at 37°C in a humidity chamber for 24 hours before being examined.

(e) Purification of immobilisation antigen

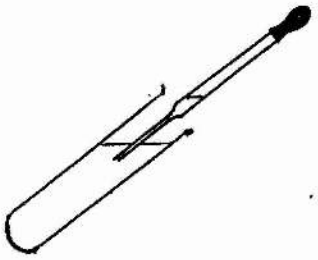
Antigen was purified as described by Jones (1965). Briefly, the dialysed salt/alcohol extract was made up to 75% saturated $(NH_4)_2SO_4$, the protein precipitate pelleted, redissolved in distilled water, dialysed against several changes of distilled water and applied to a Sephadex SPC50 column. Protein was eluted with a gradient of pH 4.2-pH 5.2 0.05M sodium acetate buffer. The cell surface antigen, which eluted at pH 4.6 - 4.8, was collected and lyophilised.

(iv) Transformation

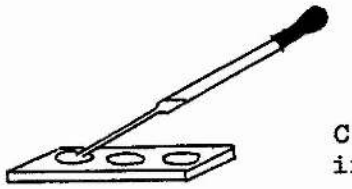
To a culture of cells in late log phase at 25°C was added an equal volume of sterile grass medium heated to 42-44°C. This increased the temperature of the culture to 32°C. It was immediately placed in a waterbath at 32°C and later transferred to an oven at 32°C. The serotype of

Fig. 1

Stages of culturing of one litre from one cell.

A 

One cell taken from stock culture

B 

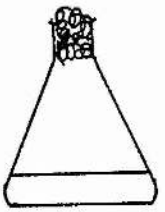
Cell transferred to 1ml bacterised medium in depression slide

C 

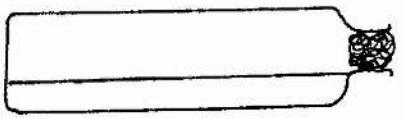
1ml culture added to 1ml diluted grass medium in a tube

D 

2ml culture made up to 10ml with grass medium

E 

10ml culture added to 100ml grass medium in 500ml flask

F 

100ml culture transferred to 2.5 litre Thompson bottle containing 1 litre grass medium

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the culture was tested at intervals by immobilisation.

3. Results and Discussion

(i) Culturing

Paramecia were routinely grown on grass infusion which was bacterised with Klebsiella aerogenes as a food source. Each litre of culture was grown from a single cell. Cultures derived from cells from the stock culture grew at a rate of 1.6 fissions per day at 25°C and 2.3 fissions per day at 32°C. These rates are higher than the corresponding fission rates of 1.2 and 1.8 per day for cultures derived from cells which had gone through a number of fissions in logarithmically growing culture, for example cells from a one litre culture. It is possible that conditions in the stock culture, i.e. low numbers of bacteria and lower than average temperature, encourage autogamy. It is well established that nuclear reorganisation makes a culture move vigorous, (Sonneborn, 1938).

Because of the variation in growth rate of a culture according to the origin of the cell from which it was derived, each litre of cells was always grown from a single cell taken from the stock culture. In this way fission rates could be standardised.

During growth of the paramecia, it was important to keep the ratio between paramecia and bacteria fairly constant. Too low a density of bacteria caused the fission rate of the paramecia to fall and eventually the culture reached stationary phase as the paramecia stopped dividing.

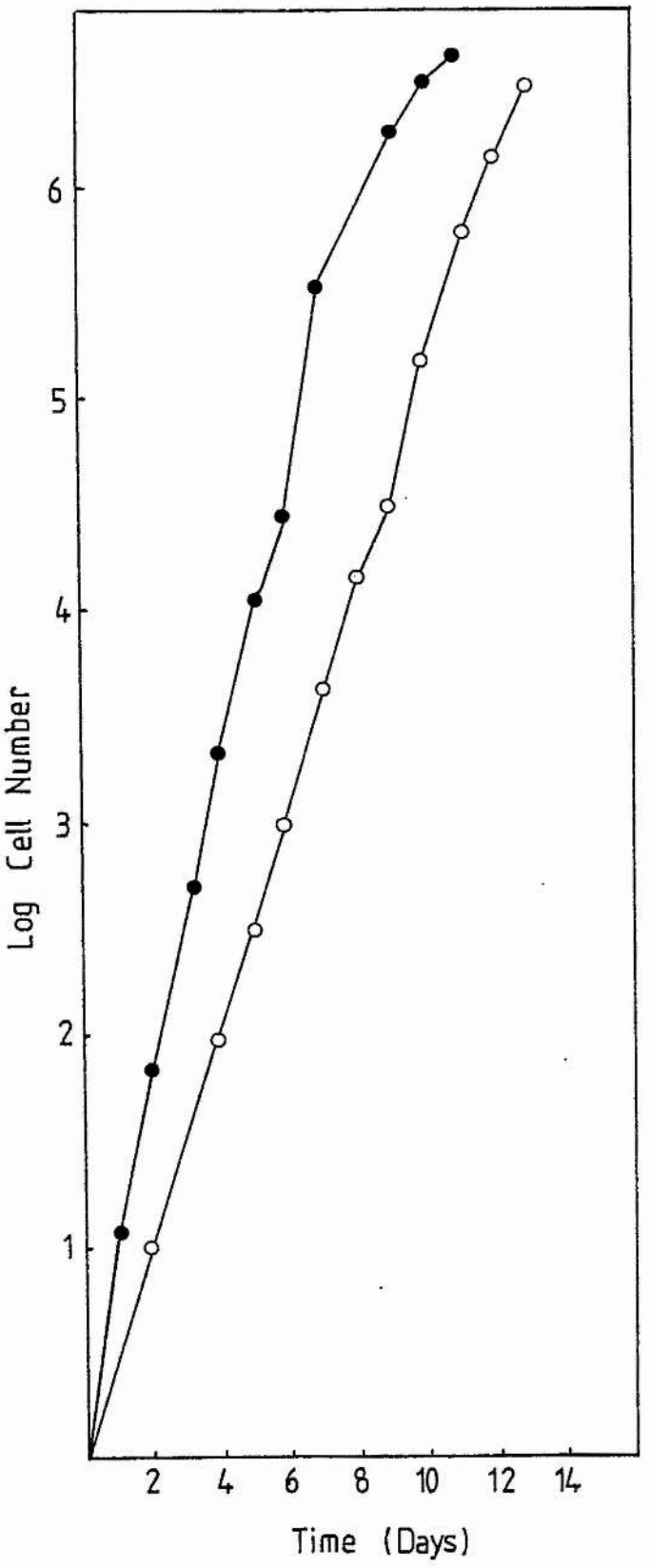

Fig. 2

Typical growth kinetics of cultures grown at 25°C and 32°C.

- ○ 25°C Fission rate 1.75/day
- ● 32°C Fission rate 2.3/day

Too high a density of bacteria again resulted in reduction of the fission rate. This was probably due to the removal of oxygen from the medium by the large numbers of bacteria. A constant fission rate during growth of the culture was achieved by increasing the culture volume gradually as shown in Fig. 1.

In this method bacteria are introduced only at the single cell isolate stage. Subsequently sterile grass is added and bacteria carried over at each stage. An alternative method used was to bacterise sterile grass medium and to adjust the pH to 7 prior to addition of paramecia at every stage of culture. This method was found to give only a slight increase in fission rates and so, being more labour intensive, was discontinued.

The growth rate of a culture of paramecia varies according to the temperature of incubation. At 25°C paramecia divided at a rate of 1.6 fissions per day on average and, at 32°C, at 2.3 fissions per day on average. (Fig. 2).

In preparations requiring large numbers of cells, the maximum density of the culture, i.e. the number of cells per litre, is more important than the fission rate. Generally the maximum density reached by paramecia grown under the conditions described is 5000 cells per ml. This density gives a yield of 0.5 ml packed cells from each litre of culture. At 32°C a similar maximum density of 5000 cells/ml is reached but, since the cells tend to be smaller than those grown at 25°C, the yield is generally

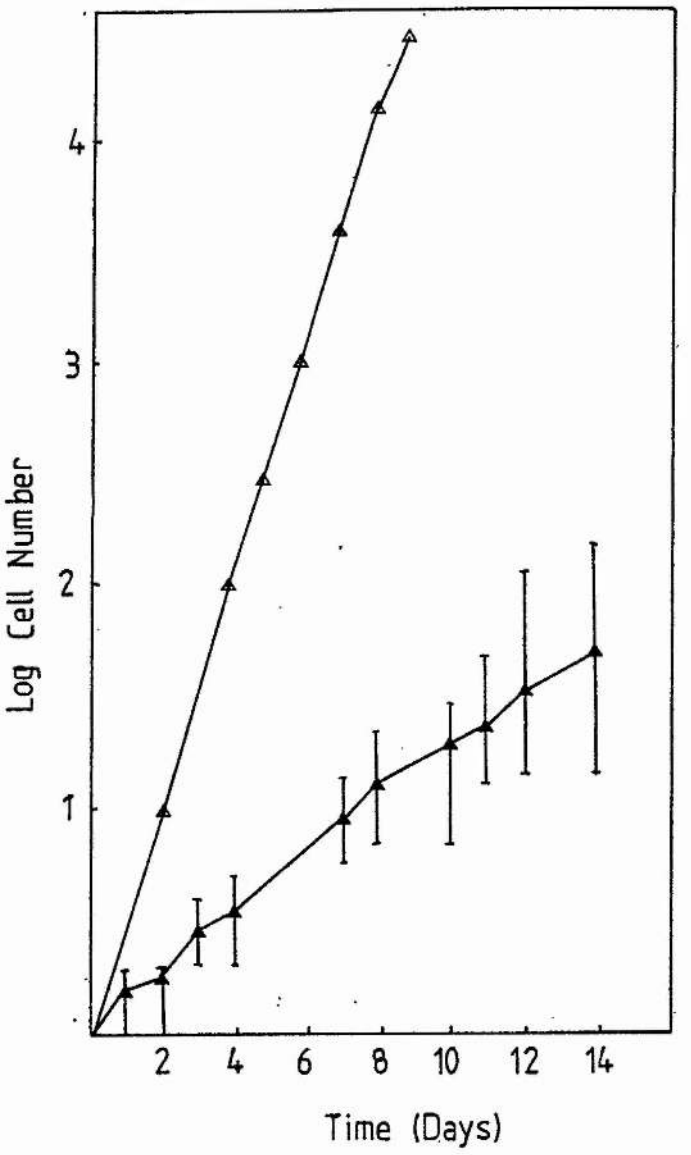

Fig. 3

Average growth pattern of six isolates in axenic medium at 25°C. The bars represent the extreme values in the sample of six.

The growth pattern of cells grown at 25°C in monoxenic medium is included for comparison.

▲ Average cell number in axenic culture at 25°C
Fission rate - 0.5 fissions/day

△ Cell number in monoxenic culture at 25°C.
Fission rate 1.75 fissions/day

0.3 - 0.4 ml packed cells per litre. Usually, however, cultures were harvested before the maximum density had been reached to ensure that the cells were actively growing. Consequently the yield of cells normally obtained was less than 0.5 ml per litre at 25°C and 0.3 - 0.4 ml per litre at 32°C. To grow one litre of cells from a single cell to the maximum density requires the culture to undergo 23-24 fissions. This takes approximately 2 weeks at 25°C and 10 days at 32°C.

(ii) Axenic Culturing of Paramecia

Paramecium can be grown in axenic medium but, unlike the related ciliate Tetrahymena, does not grow well. The axenic medium is semi-defined (Van Wagtendonk and Soldo, 1970). Initially it was hoped that a culture of stock 168 could be established in axenic medium and that this could be used for labelled metabolite incorporation studies, avoiding the problems of simultaneous bacterial labelling. However, the growth rate varies according to the stock used. Stock 168, after transfer to axenic medium grew at a rate of 0.3-0.5 fissions per day at 25°C (Fig. 3). This was less than one third of the growth rate of a monoxenic culture grown at the same temperature. The lengths of time required to obtain large numbers of cells and the constant risk of infection, made the use of axenic cultures impracticable.

(iii) Preparation of Antisera

Antisera were routinely prepared by injection, into

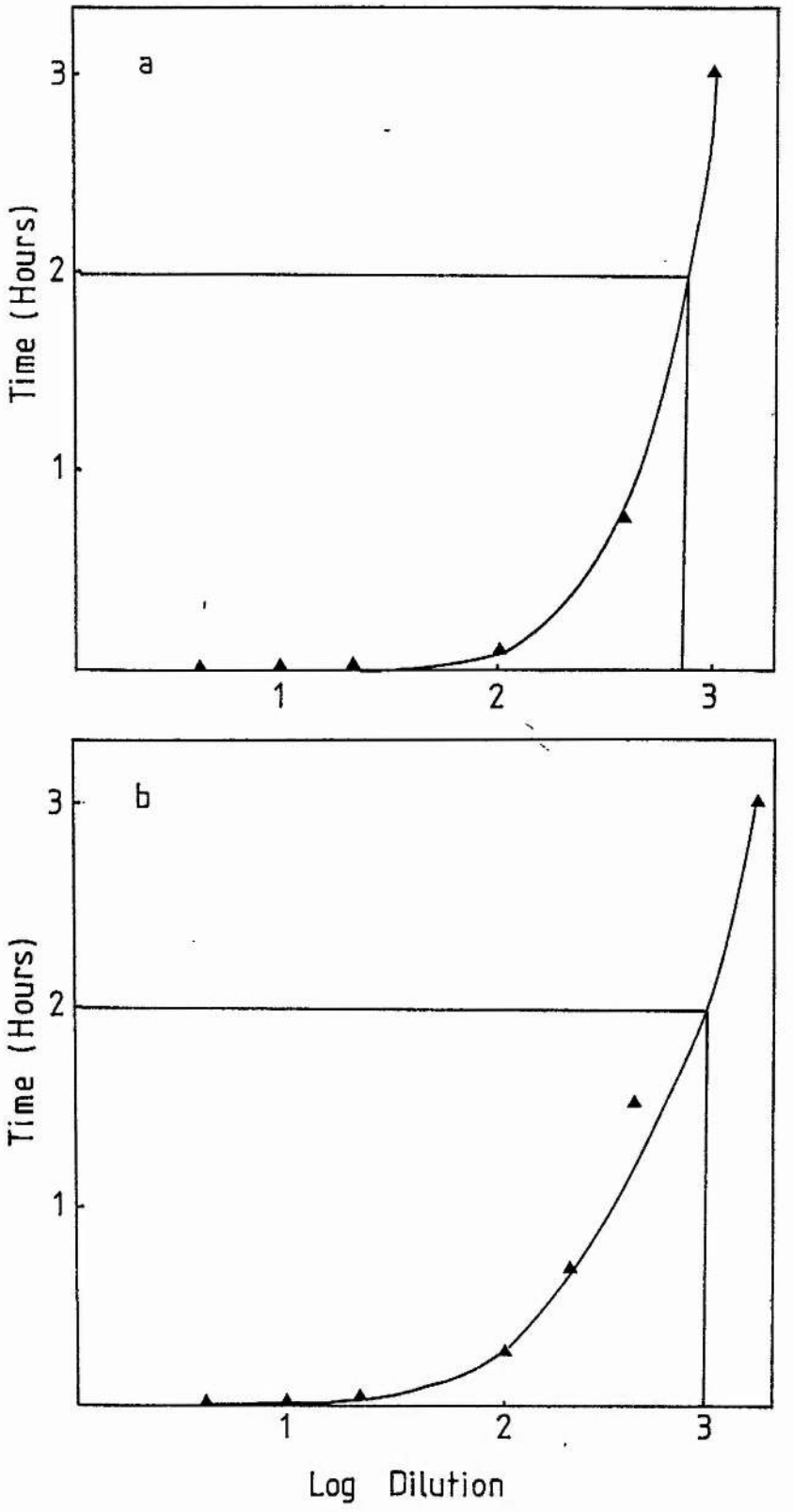

Fig. 4 a and b

Times of immobilisation of G cells with various dilutions of Antiserum from rabbit G2.

a Antiserum taken from rabbit G2 4 weeks after injection of antigen. Titre = 800.

b Antiserum taken from rabbit G2 6 weeks after injection of antigen. Titre = 1000.

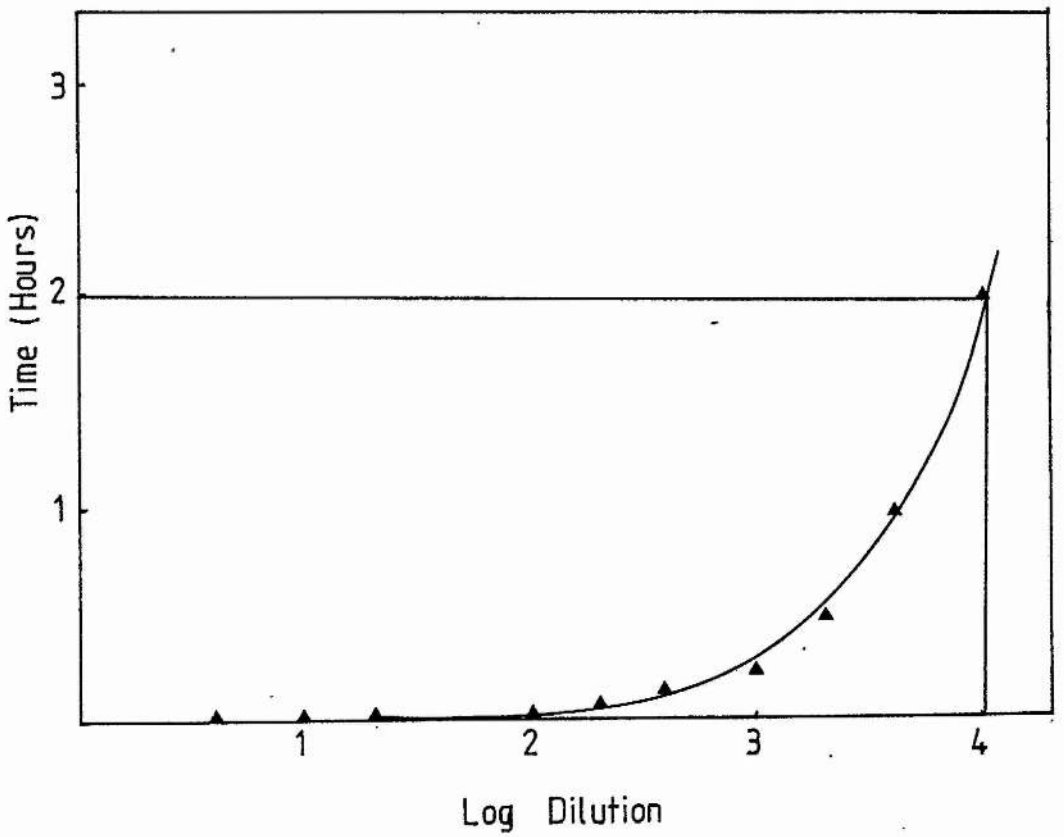

Fig. 5

Times of immobilisation of D cells with various dilutions of Antiserum from rabbit D2 6 weeks after injection of Antigen. Titre = 10,000

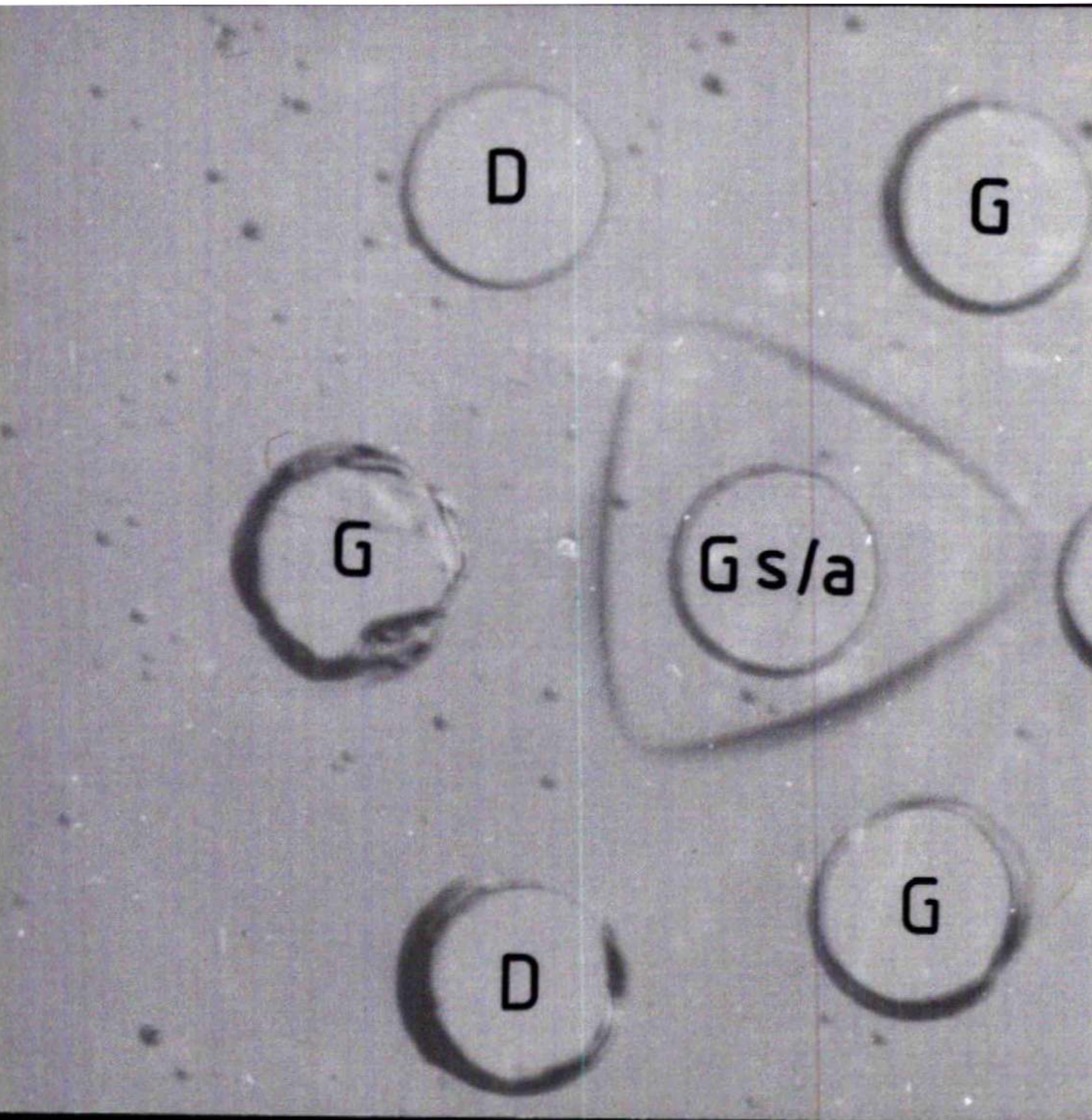

Fig. 6

Fig. 6

Immunodiffusion of a sample of salt/alcohol extract of G cells in the central well against samples of anti-G and anti-D sera in the surrounding wells. Precipitin arcs have formed only between the G salt/alcohol extract and anti-G serum indicating no cross-reaction of anti-D serum with G antigen.

G s/a - salt/alcohol extract of G cells

G - anti-G serum

D - anti-D serum

a rabbit, of either a crude salt/alcohol extract of paramecia grown either at 25°C or 32°C, or a solution of cell surface antigen protein purified from this extract. This was injected subcutaneously as a multiple emulsion. Usually a booster injection of the same emulsion was given a few weeks later. Six weeks or more after the injection, a sample of blood was removed from the marginal ear vien and the titre estimated by making a series of standard dilutions which were used to immobilise cells of the appropriate serotype. The serum titre was defined as the dilution of serum which would completely immobilise cells in 2 hours.

The serum titre increased with time in the rabbit and finally reached a plateau. Serum from rabbit G2 was tested 4 weeks after the initial injection and was found to have a titre of 1:800. By 6 weeks the titre had risen to 1:1000 (Fig. 4a and b). D antiserum consistantly had a higher titre than G antiserum. One batch had a titre of 1:10,000 (Fig. 5). The reason for the apparent higher antibody stimulating capacity of the D antigen is unknown and is surprising in view of the structural similarity of the two antigen proteins (Jones, 1965., Steers, 1965.).

None of the antisera prepared showed any cross-reaction as tested by immobilisation, even at high concentration. Specificity was also tested by double-diffusion in agar against the salt/alcohol extract from cells of the heterologous serotype. Again no cross-reaction was observed, even with undiluted serum (Fig. 6).

4. Summary

With regard to culturing, *Paramecium* is not as convenient as the related ciliate *Tetrahymena*. Growth rates and maximum densities are much lower, necessitating longer culture times and larger volumes to obtain the amount of cells required for the extraction of certain cellular components. Although certain stocks of *Paramecium* grow well in axenic medium, stock 168 is not one of them, having a growth rate one third of that of monoxenic cultures. Because of this the use of axenic cultures was impracticable. Stock 168 has been cultivated both at 25°C and 32°C. At these temperatures the G and D serotypes are expressed respectively. By increasing the temperature from 25°C to 32°C the G serotype can be rapidly transformed to the D serotype. The two serotypes can be detected by their reaction to antisera, which are easily prepared by injection of an extract into a rabbit. Using an appropriate dilution of antiserum, the serotype of a culture of cells could be rapidly determined by the immobilisation test.

CHAPTER II

Organisation of Macronuclear DNA

1. Introduction

Ciliates possess two types of nuclei, macronuclei and micronuclei (reviewed by Raikov, 1976). The micronucleus is the genetic nucleus of the cell. It is, however, synthetically inactive, synthesising little or no RNA (Gorovsky and Woodward, 1969). RNA synthesis is primarily carried out by the macronucleus, which is a large polyploid nucleus. In contrast to the micronucleus, it plays no part in meiotic events, disintegrating prior to cell conjugation, later to be formed anew from a micronuclear division product in the exconjugant cells.

A number of differences have been observed, not only in the function of these two types of nuclei, but also in the characteristics of their DNA. For instance, differences have been reported in the types of histones associated with the DNA (Gorovsky et al., 1977), in the DNA-dependant polymerase activities (Tait and Cummings, 1975) and in the structure and number of ribsomal genes (Yao et al., 1974).

The most striking differences between the two types of nuclei found in ciliates is observed in the hypotrichs Stylonichia (Ammermann et al., 1974) and Oxytricha (Lauth et al., 1976) and a brief description is pertinant since it illustrates one of the mechanisms for the formation of the macronuclear genome.

The micronuclear DNA from hypotrichs, in a number of respects, is similar to that of higher eucaryotes. It is of high molecular weight, has a wide range of GC values and a number of satellite DNAs are in evidence. This DNA renatures over a broad range of $C_o t$ values indicating that it contains a considerable amount of satellite and middle-repetitive DNA. Slowly renaturing DNA, presumably consisting of uniquely represented sequences, has a complexity some 500 times higher than that of Escherichia coli and therefore falls within the range of complexity found in many higher eucaryotes (Britten and Kohne, 1968., Laird, 1971).

However, hypotrich macronuclear DNA is quite different from micronuclear DNA. Firstly, it is of low molecular weight, the DNA falling within a limited size range of 1000-20,000 nucleotides (Swanton et al., 1980) and apparently has been cleaved at specific sites (Lawn et al.,1977., Elsevier et al., 1978., Lipps and Steinbruck, 1978). Secondly, it has a restricted density spectrum, only one density component being found (Bostock and Prescott, 1972). Thirdly it reassociates very simply with simple second order kinetics indicating that there is little or no repetitive DNA (Prescott and Murti, 1973). Furthermore, the complexity is only some 13 times higher than that of E. coli.

Since the macronucleus develops from a micronuclear division product, one must conclude that there is considerable loss, or gross underrepresentation of many DNA sequences during that development. Coincident with macronuclear development are many microscpoic changes, including a polytene

chromosome phase (Rao and Ammermann, 1970., Murti, 1973). These developmental changes have only been described in the hypotrichous groups of ciliates.

Is this DNA sequence diminution a charcteristic feature of macronuclear development in the ciliates? Examination of this question in Tetrahymena has led to the conclusion that it is not. In Tetrahymena, macronuclear DNA is very similar to that of the micronucleus and it is estimated that, if there is any DNA sequence loss during macronuclear development, this is less that 10% of the micronuclear DNA sequences. (Yao and Gorovsky, 1974) in contrast to the value of 98% in the hypotrichs.

Paramecium is more closely related to Tetrahymena than to the hypotrichs. This, together with the absence, like Tetrahymena, of a polytene chromosome phase during macronuclear development (Jurand et al., 1964) leads one to the prediction that, again like Tetrahymena, there should be little or no sequence loss during macronuclear development in Paramecium.

It has proved impossible to make a direct comparison between macronuclear DNA and micronuclear DNA in Paramecium due to the difficulty in isolating large amounts of micronuclei free from macronuclei or macronuclear fragments (Cummings and Tait, 1975). However, the macronuclear DNA from a variety of stocks and species of Paramecium has been examined by a number of authors (Allen and Gibson, 1972., Soldo and Godoy, 1972., Cummings, 1975). Unfortunately there appears to be some disagreement about many of the

properties of this DNA. This may be due to different extraction techniques, to genuine differences in the DNA of different stocks and species or to a combination of both factors.

Since the DNA of stock 168 of Paramecium primaurelia was to be used to estimate the number of genes being transcribed under different environmental conditions, it seemed essential to chracterise the DNA of stock 168 in terms of GC content, length and sequence complexity to provide a background to work on gene expression. In addition it was hoped to tackle the question of whether or not there was reduction of sequence complexity during macronuclear development in Paramecium.

DNA can be isolated either from intact cells or from purified macronuclei. For this work, DNA was isolated from purified macronuclei for three reasons. Firstly, Allen and Gibson (1971) note an effect of culture conditions, i.e. presence and amount of bacteria, on the density of the DNA isolated from intact cells. This suggests that DNA isolated from cells grown in bacterised medium is contaminated with bacterial DNA. Secondly, Paramecium has many mitochondria which contain large amounts of DNA relative to the mitochondria of other organisms (Flavell and Jones, 1971). It has been established that 10% of the cellular DNA is mitochondrial in origin (Soldo and Godoy, 1972). Thirdly, if there is any difference in DNA sequences between the macronuclei and micronuclei, it

is obviously preferable to restrict analysis of RNA synthesis to that transcribed from macronuclear DNA alone rather than from a mixture of macronuclear and micronuclear DNA.

2. Materials and Methods

(i) Preparation of Macronuclei

The method used was that described by Cummings and Tait (1973) which involves treatment of the cells with nonionic detergents in the presence of calcium ions and spermine. The cells are then homogenised and the macronuclei purified by a brief centrifugation through 2.1M sucrose, containing 20% glycerol.

(ii) Preparation of DNA from Macronuclei

The nuclear pellet was resuspended in 4ml of 20mM Tris HCl, disodium ethylenediaminetetracetic acid 1mM (EDTA), 10mM NaCl, pH 7.8 and stirred at $4^{\circ}C$ for 15 minutes. The solution was made 0.5% sarkosyl (Ciba-Geigy) added gradually, and stirred for a further 30 minutes. It was then adjusted to 1M $NaClO_4$ and 2ml of "phenol" added ("phenol" is redistilled phenol containing 14% cresol, 0.1% hydroxyquinoline and saturated with distilled water). The mixture was stirred at room temperature for 15 minutes and 2ml of "chloroform" added ("chloroform" is chloroform mixed with isoamyl alcohol in the ratio 22:1). The phases were separated by centrifugation and the aqueous phase reextracted with phenol/chloroform (mixed in a 1:1 ratio) and again with chloroform before

being precipitated by the addition of two volumes of absolute alcohol. DNA was spooled from the interphase, dried and redissolved in 1-5ml ribonuclease buffer (0.1M NaCl, 0.01M Tris HCl, 0.01M EDTA, pH 7.5). The DNA solution was made 100 µg/ml ribonuclease A (Sigma) and digested at 37°C for 2 hours. Enough 20% SDS was added to give a final concentration of 0.5% and the solution was adjusted to 0.1mg/ml proteinase K (Boehringer) Digestion was continued for 2 hours at 37°C and the solution phenol/chloroform extracted. The aqueous phase was chloroform extracted and precipitated with two volumes of alcohol. The DNA was spooled out, dried, redissolved in an appropriate buffer, either SSC or TE, and dialysed against the same buffer before being stored at 4°C. The concentration of the DNA solution was measured in a Pye-Unicam dual beam spectrophotometer monitoring at 260nm.

(iii) Characterisation of Macronuclear DNA

(a) Buoyant density of DNA

The buoyant density profile of DNA in CsCl was measured in a M.S.E. centroscan centrifuge. A sample of DNA from Micrococcus lysodeikticus was included as a density marker.

(b) Thermal melting profile

The DNA solution to be melted was dialysed against SSC and diluted to less than 1OD. The DNA solution and reference solution were degassed, loaded into 1ml quartz cells and overlaid with paraffin oil. The DNA solution was melted in a Pye-Unicam dual-beam spectrophotometer with a

heating programme. The sample was allowed to equilibrate at 30°C for 15 minutes and the temperature subsequently raised to 100°C at a rate of 0.5°C/min. The OD was recorded every 30 seconds on a strip chart recorder. A sample of E. coli DNA (Boehringer) was similarly treated, as a standard for comparison.

(c) Determination of the length of macronuclear DNA by alkaline sucrose gradient centrifugation

100µl of DNA at a concentration of 50µg/ml or more was made up to 0.1 M NaOH and incubated at 37°C for 15 minutes. It was then layered on a 12ml gradient of 5-20% sucrose in 0.9M NaCl, 0.1M NaOH. This gradient was centrifuged in parallel with a gradient overlaid with 100µl of marker DNA (a Hind III digest of λ DNA obtained from Boehringer). The gradients were centrifuged in a M.S.E. 6x14ml titanium swing-out rotar in a M.S.E. superspeed 65 centrifuge for 14 hours at 25 k r.p.m. at 10°C. The tubes were removed, pierced and pumped at low speed through a L.K.B. flow cell recording at 260nm. The OD profile was recorded on a chart-recorder.

(d) Electron-microscopy of DNA

DNA was prepared for electron microscopic examination by the method described by Trendelenburg et al. (1976). Photomicrographs were made of 25µm^2 areas of spread DNA and all molecules within this area were measured using an electronic digitiser programmed for contour length analysis.

(e) Restriction digestion and agarose gel electrophoresis of DNA

Two enzymes were used to digest the DNA, EcoRI and Hind III (Boehringer). 1-5µg of DNA was digested with 1-5 units of enzyme for 1 hour at 37°C in a 20-50µl reaction volume. In the case of EcoRI the conditions used were 0.1M Tris HCl, 50mM NaCl, 10mM $MgCl_2$, pH 7.5. For Hind III the conditions used were 10mM Tris HCl, 50mM NaCl, 10mM $MgCl_2$, 14mM DTT, pH 7.6. At the end of the reaction time the samples were cooled and 5µl of 1% bromophenol blue in 50% sucrose added. The samples were loaded on a 30 x 30cm, 3mm thick 1% agarose gel made up in electrophoresis buffer (0.036M Tris, 0.03 M NaH_2PO_4, 0.01M EDTA pH 7.6). The gel was run at 20V for 20-24 hours or until the bromophenol blue marker was 1cm from the bottom of the gel. After electrophoresis, the gel was removed and immersed for 30 minutes in electrophoresis buffer containing 1µg/ml ethidium bromide. The gel was fluoresced with UV light and photographed using a Polaroid MP4 land camera with a red filter.

(IV) Renaturation Kinetics of Macronuclear DNA

(a) Optical renaturation in the spectrophotometer

To 50µg of sonicated DNA in 0.8ml of TE buffer was added 0.1ml of 1M NaOH. The mixture was incubated at 55°C for 15 minutes and transferred to a quartz cell in a spectrophotometer preheated to 55°C. 0.1ml of 2M NaH_2PO_4 was added and the OD recorded every minute from the point of mixing for the first hour and subsequently every 5 minutes

for the next twenty hours.

(b) Optical renaturation of DNA

Needle-sheared DNA at 1mg/1ml in 0.12M PB was sealed in 50µl aliquots in 100µl chromic acid-treated capillary tubes. Two samples (native samples) were placed in a waterbath at 55°C. The remainder were boiled for 5 minutes, two removed and placed immediately into iced water (zero samples). The rest were placed in a 55°C water-bath, samples removed at intervals up to 100 hours and stored at 0°C. After all the samples had been taken, they were diluted to 1ml in 0.12M PB, allowed to equilibrate at 37°C and the OD measured in a spectrophotometer preheated to 37°C. The samples were then heated to 95°C and the OD re-read. The hyperchromicity of each sample was determined and compared with the hyperchromicity of the two native samples. As a standard, a sample of E. coli DNA was similarly treated.

(c) Iodination of DNA

DNA solution was diluted to a concentration of 10-20µg/ml in 0.1M ammonium acetate buffer pH 5.0. This was sonicated at 20 watts in 3 one minute bursts using a 3mm tip at 0°C. The DNA solution was denatured by heating for 5 minutes in a 100°C water-bath. 0.4ml of this solution was iodinated with I^{125} by the method of Commerford (1971).

The reaction mix was applied to a 15cm x 1cm Sephadex G50 column equilibrated in 0.12M PB The void volume of

the column had previously been determined by eluting a sample of 10% blue dextran (Pharmacia). 2ml of the sample eluate was collected in the void volume and stored at $4^{o}C$.

(d) Renaturation of DNA with iodinated DNA

DNA was sonicated as above and diluted to a concentration of 1mg/ml in 0.12M PB. Iodinated DNA was added to a concentration of 1µg/ml and 25ul samples were sealed in 50µl chromic-acid treated capillaries. Samples were then denatured and renatured as described for optical renaturation. When all the samples had been taken, they were diluted to 200µl in S_1 buffer (0.03M sodium acetate, 3×10^{-5}M $ZnSO_4$, 0.01M NaCl, pH 4.5) 100ul was removed from each sample and pipetted on to a 2.5cm Whatman paper filter, which had been previously washed in 1mg/ml Bovine serum albumin (BSA) (Sigma, Fraction V). To the remaining 100ul, 10µl of single-strand-specific S_1 nuclease (Boehringer) was added and the sample was incubated at $45^{o}C$ for 30 minutes. After this time the samples were pipetted on to BSA washed filters. All filters were washed five times in 500ml 5% TCA, twice in absolute ethanol and one in diethyl ether. They were then dried and counted for one minute in 5ml toluene-based scintillation cocktail (Nuclear Enterprises, NE 233).

(e) Melting profile of labelled DNA on hydroxyapatite

A sample of labelled DNA containing at least 2000 cpm was diluted in 1ml 0.05M PB and applied to a 1ml hydroxyapatite

(HAP) column consisting of a pasteur pipette within a circulating water-jacket connected to a Haake water-bath and pump. The temperature could be accurately varied to within 0.5°C. The column was equilibrated in 0.05M PB at 50°C. The wash-through from the sample was collected and the column washed with 0.12M PB. The temperature was increased in 2°C increments from 50°C to 100°C and at each temperature the column was washed with 2ml of 0.12M PB preheated to the same temperature. The eluate at each stage was collected in scintillation vials and dried down to 0.5ml or less in a 100°C oven. 5ml dioxan-based scintillation cocktail (Nuclear Enterprises, NE250) was added and the samples were counted in a gamma scintillation counter.

3. Results

(i) Isolation of Macronuclei

Macronuclei were isolated from paramecia in order to obtain a pure preparation of macronuclear DNA free from mitochondrial DNA, micronuclear DNA and bacterial DNA.

The method of Cummings and Tait (1975) was used. This is a modification of an earlier procedure developed by Cummings (1972).

The isolation procedure involved treating the cells at 0°C with the non-ionic detergents Nonidet P40 and sodium deoxycholate. This causes the cells to swell (Fig. 7), and become fragile and therefore more easily homogenised. A small percentage of the cells lyse under

Fig. 7

A phase contrast micrograph of paramecia incubated in nuclear homogenisation buffer showing the charcteristic round shape. The cell at the top of the photograph has lysed and is releasing its macronucleus (Ma) into the buffer.

(Magnification x 230)

Fig. 8

A phase contrast micrograph of paramecia macronuclei after centrifugation through dense sucrose. The macronuclei (Ma) are contaminated by highly refractile crystalline inclusions (CI)

(Magnification x 230)

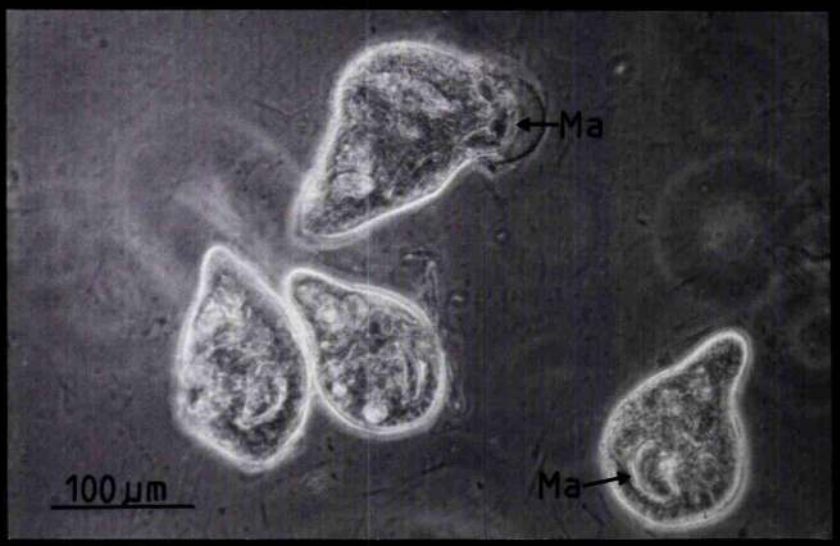

Fig. 7

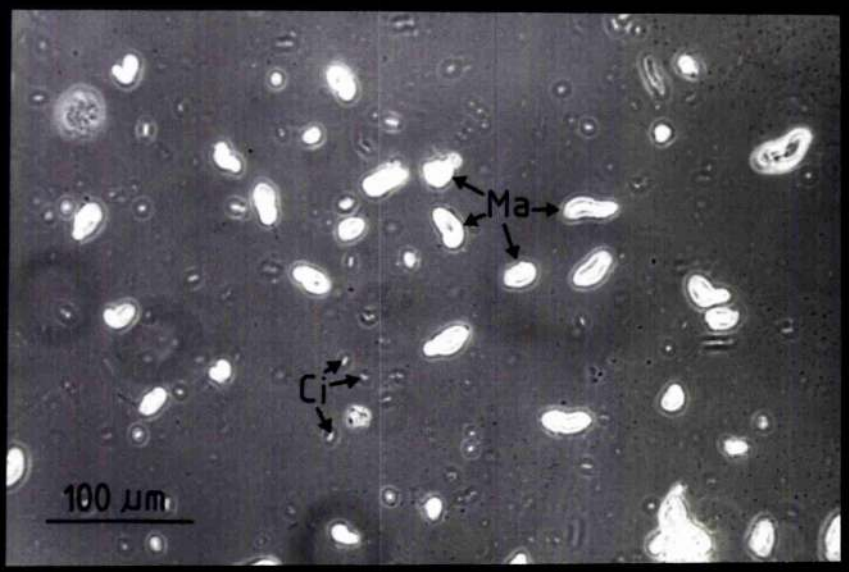

Fig. 8

these conditions, freeing the macronuclei into the buffer (Fig. 7). The presence of calcium ions and spermine in the buffer preserves the shape of the nuclei and the majority show the characteristic kidney shape of *in vivo* nuclei (Fig. 8).

The cells were homogenised as soon as some had began to lyse and release their nuclei with the buffer. It was found that the optimum time was 10-15 minutes after the addition of the buffer rather than the 20-25 minutes recomended by Cummings and Tait (1975), unless the buffer had not been freshly made up, in which case a longer period of incubation was required.

The macronuclei were purified from the homogenate by brief centrifugation through a pad of 2.1M sucrose containing 20% glycerol (Fig. 8). Recovery was generally less than 50%, probably due to trapping of macronuclei in the interphase. The pelleted macronuclei show a high degree of purity, being contaminated only with crystals of characteristic size and shape but of unknown composition. Most importantly, no bacteria are seen in the macronuclear preparation. A longer centrigation improved the yield of macronuclei slightly. However, some membranous material, particularly oral grooves, also pelleted. Macronuclei were generally isolated from cells grown at 25°C since it was more convenient to grow large volumes of culture at this temperature. However, DNA isolated from macronuclei of cells grown at 32°C was identical in every parameter investigated to DNA from macronuclei of cells grown at 25°C.

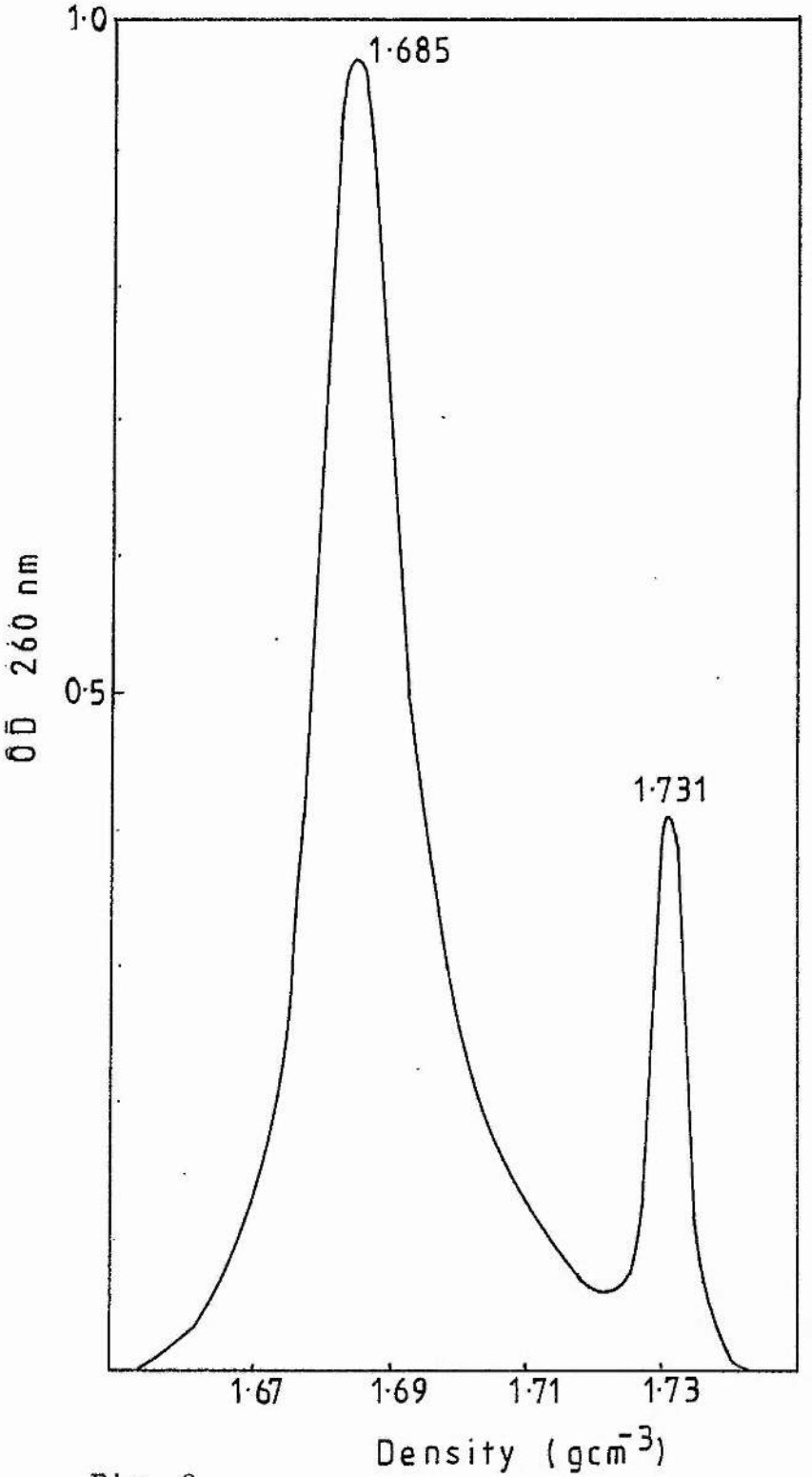

Fig. 3

Buoyant Density Centrifugation of Paramecium macronuclear DNA in a CsCl gradient. Micrococcus lysodeikticus DNA (density 1.731 gm cm^{-3}) was included as a density marker.

(ii) Preparation and Purification of DNA from Macronuclei

The macronuclei were lysed in a low salt solution containing EDTA. The ionic detergent Sarkosyl was added to dissociate protein from DNA and the nucleic acid purified by phenol extraction. On precipitation with alcohol, DNA could generally be spooled out on a glass rod suggesting that it was of quite high molecular weight. However, in some preparations it was not possible to spool out the DNA due either to low concentrations or to breakage of the DNA during preparation. Failure to spool out the DNA was experienced most frequently when nuclei were isolated from stationary phase cells and may be related to higher levels of nuclease in stationary as opposed to log-phase or late log-phase cells. This agrees with the work of Cummings (1975), who finds that DNA from stationary phase cells is shorter, on average, than DNA from log-phase cells.

RNA was removed from the wound-out material by digestion with pancreatic ribonuclease. Ribonuclease and residual protein were removed by proteinase K digestion followed by a second phenol extraction.

DNA was generally dissolved and stored in SSC buffer. When a sample was to be iodinated it was dissolved and stored in TE buffer since the citrate ion appears to be inhibitory to the iodination reaction.

(iii) GC Content of Macronuclear DNA

(a) Buoyant density of macronuclear DNA in CsCl

The buoyant density of macronuclear DNA was examined

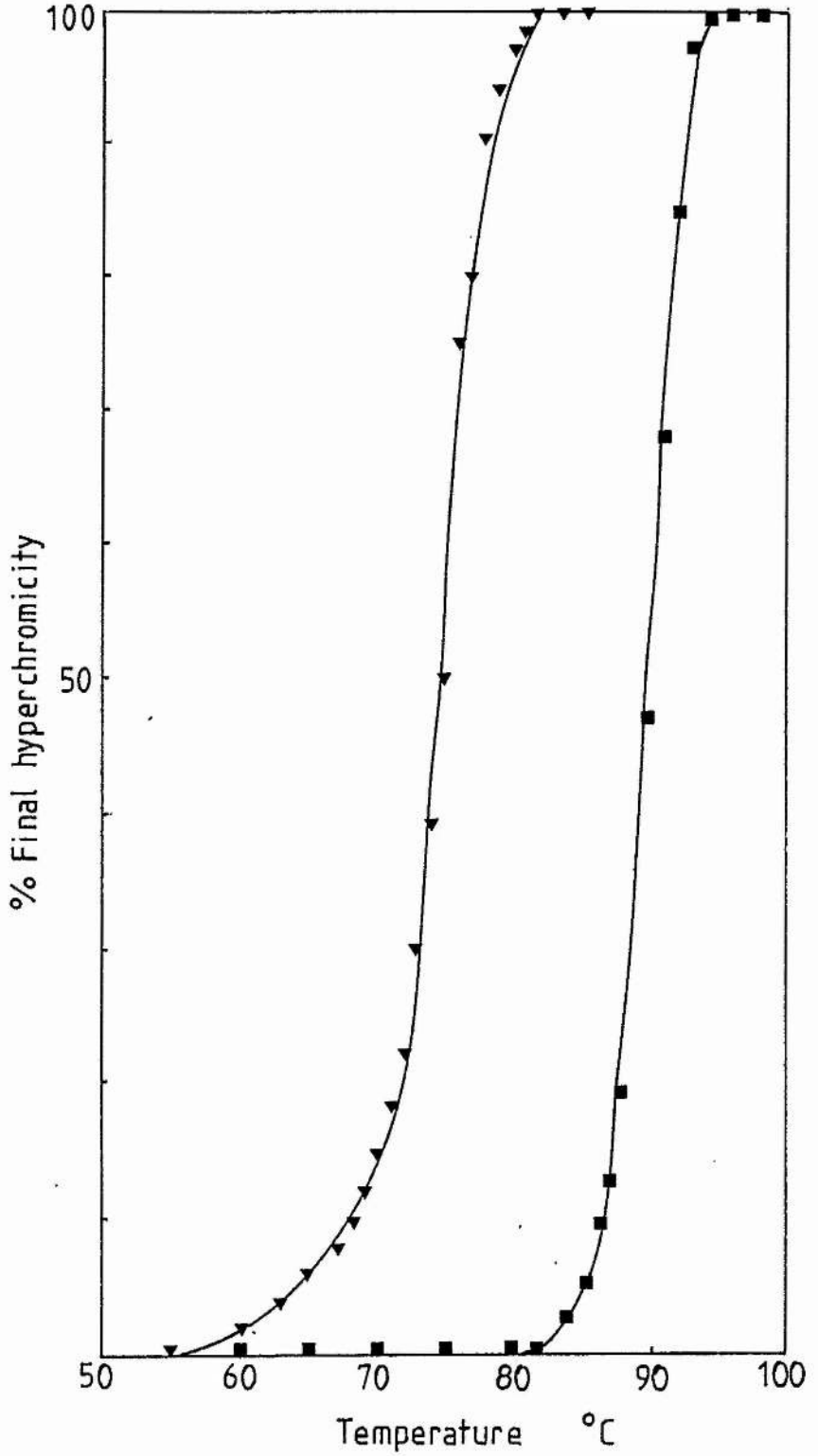

Fig.10

Thermal dissociation of Paramecium macronuclear DNA and E. coli DNA.

▼ Macronuclear DNA

■ E. coli DNA

on a CsCl gradient. DNA from Micrococcus lysodeikticus was included as a density marker of 1.761g cm^{-3}. Macronuclear DNA had a simple density profile, showing no satellite peaks or shoulders (Fig. 9). The average density is 1.685g cm^{-3} which indicates a GC content of 25.5% (Mandel et al.,1968). This value is within the range of 21-26% found for P. primaurelia stock 168 by Cummings (1972). Allen and Gibson (1971) find the higher value of 28% GC for 6 different stocks of P. primaurelia. Such a high value may be related to the extraction of DNA from whole cells rather than from purified macronuclei.

(b) Thermal melting profile of macronuclear DNA

Macronuclear DNA was melted in SSC in parallel with a sample of E. Coli DNA. Macronuclear DNA melts with a Tm of 76°C(Fig. 10). This indicates a GC content of 22.5% (Mandel and Marmur, 1968), and is lower than the value derived from the buoyant density. However, the relationship between Tm and GC deviates from linearity below 30% GC. Therefore, the value of 25.5%GC derived from the buoyant density is probably a more accurate estimate.

Macronuclear DNA melts with a simple profile over a narrow temperature range. This indicates that the majority of the DNA has a very similar GC content.

(iv) Size of DNA

(a) Alkaline sucrose gradients

On an alkaline sucrose gradient, macronuclear DNA has a broad size distribution with a peak at 1.6μm and the

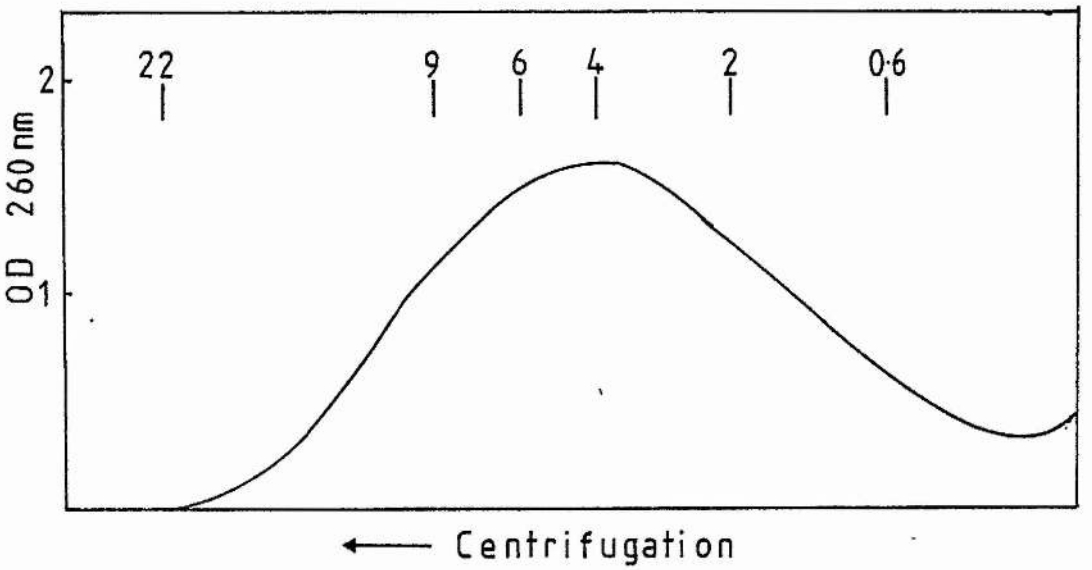

Fig. 11

Size distribution of DNA in an alkaline sucrose gradient. The gradient was centrifuged for 22 hours at 25 krpm at 10°C before being fractionated through a flow-cell monitoring at 260 nm.

A sample of Hind III restriction fragments of λ DNA (Boehringer) was centrifuged on an identical gradient run in parallel to act as markers. The values shown are the sizes of the fragments in kilobases.

Fig. 12

Photomicrograph of spread macronuclear DNA.

The bar represents 1 μm

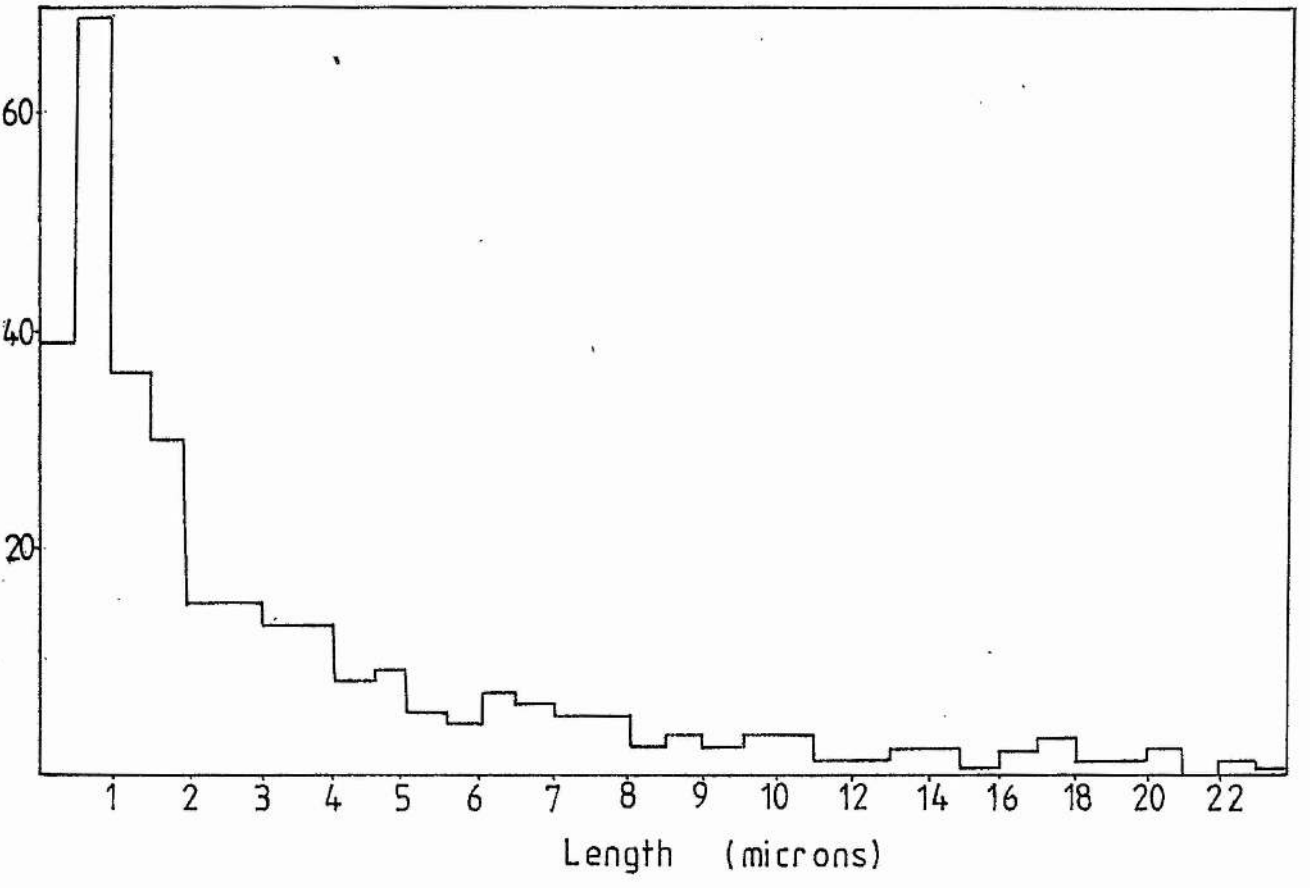

Fig. 13

Length distribution of macronuclear DNA. Photomicrographs were made of 25 μm^2 areas of spread DNA and all molecules within this area were measured using an electronic digitiser programmed for contour length analysis.

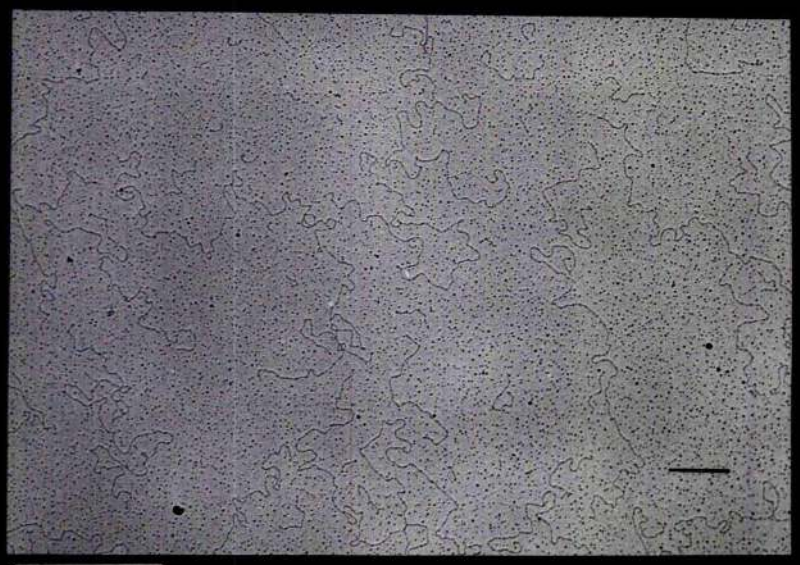

Fig. 12

majority of the DNA having lengths of between 0.05 and 6μm (Fig.11). DNA samples run on an identical neutral gradient have a similar size distribution indicating that there is little single-strand nicking of the DNA during isolation.

(b) Electron microscopy

The size distribution of the DNA as determined by alkaline gradient centrifugation is confirmed by an electron-microscopic examination of the DNA. A sample was spread by the Kleinschmidt technique (Kleinschmidt, 1968), shadowed with platinum/palladium and the electron microscope grids examined (Fig. 12). The lengths of molecules were determined by analysing photographs of random areas of the grids using an electronic digitiser programmed for contour length analysis (Fig. 13).

Molecules of up to 22μm in length were observed. However molecules as long or longer than 20μm were frequently spread over a larger area than that being analysed. This problem was reduced by overlapping several photographs in an attempt to include such molecules in the analysis, but there is clearly a measurement bias against very long molecules. There is also a measurement bias against very short molecules. At the magnification used, molecules of less than 0.1μm in length were difficult to distinguish from the background and some may have been omitted from the analysis.

Fig. 14

Fig. 14

1% Agarose gel electrophoresis of Paramecium macronuclear DNA.

In track 1 λ DNA has been digested with the restriction endonuclease HindIII and in track 2 with EcoRI In track 3 Paramecium macronuclear DNA has been digested with HindIII and in track 4 with EcoR1. Track 5 contains native Paramecium macronuclear DNA. The molecular weights of the digested λ fragments are indicated.

However, in general, this sort of analysis agrees with the alkaline sucrose sedimentation data. Macronuclear DNA has a broad range of size, but the majority is relatively short, the peak in the histogram corresponding to 0.5-1μm in length. The weight-average value is of course higher and corresponds to 3μm. The size distribution found here is similar to that observed by Cummings (1975) for P. primaurelia Stock 513. He obtains a somewhat broader size distribution with more long molecules and fewer very short molecules. This may be related to the differant extraction technique.

The size distribution of Paramecium macronuclear DNA is broader than that observed in the hypotrichs (Wesley, 1975). It is more likely that the size distribution in Paramecium is due to non-specific endogenous nuclease activity rather than to the specific cleavage which occurs in the hypotrichs.

(c) Agarose gel electrophoresis of DNA

Specific DNA cleavage in the hypotrichs gives rise to a banded pattern in agarose gel electrophoresis of macronuclear DNA. (Swanton et al., 1980). Non-specific endogenous nuclease activity might be expected to give rise to a continuous distribution of DNA rather than to distinct bands. A continuous distribution is indeed what is observed in a gel of paramecium macronuclear DNA i.e. no bands are seen (Fig. 14). The size range is roughly 2,000 - 18,000kdaltons. which corresponds to lengths of 1-9μm. This is higher than the range obtained by alkaline gradient centrifugation and

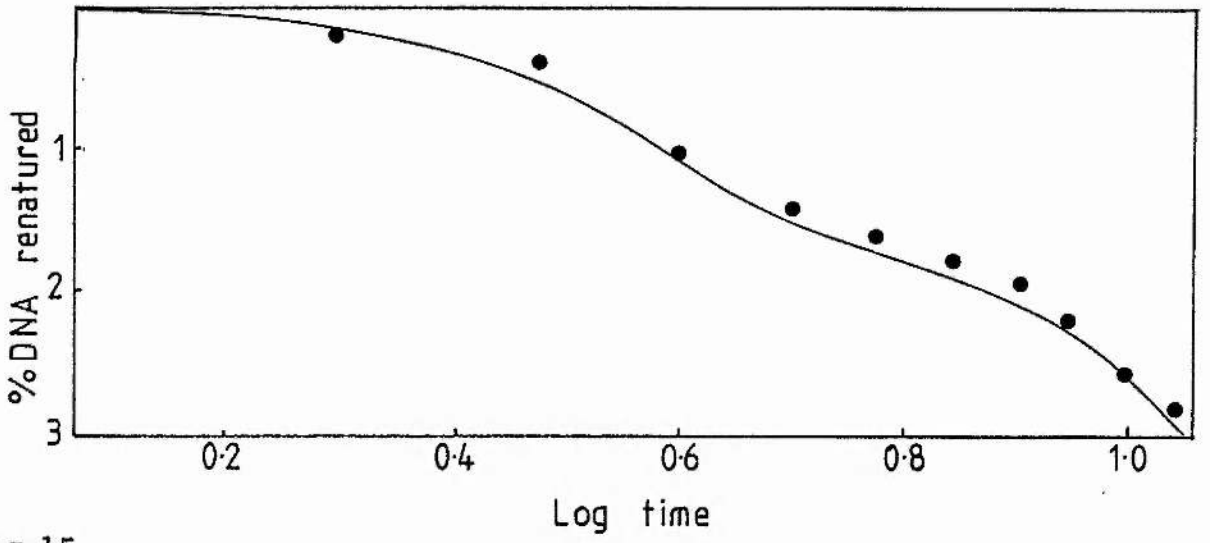

Fig.15

Rapid reassociation of macronuclear DNA. The sample was alkaline denatured and, after 15 mins incubation at 55°C, was neutralised and the extent of reassociation followed by spectrophotometry for a period of 12 hours.

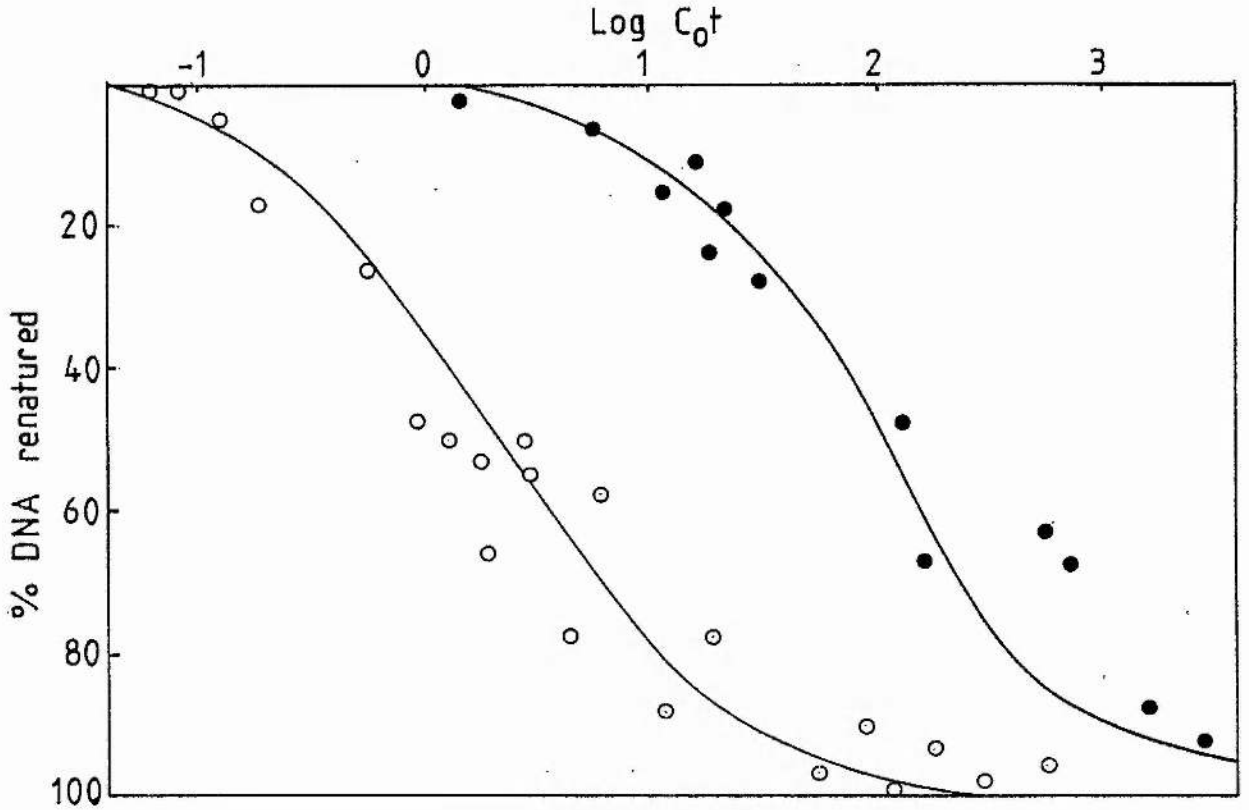

Fig. 16 Optical reassociation of Paramecium macronuclear DNA and E. coli DNA. The extent of reassociation was measured as the percentage of the native hyperchromicity of each sample.

● - Paramecia macronuclear DNA

○ - E. Coli DNA.

electron microscopy and reflects the variability in DNA size distribution between different preparations.

Restriction digestion of this DNA with the restriction endonucleases EcoRI and Hind III both give an extended smear rather than a banded pattern suggesting there are no regions of the macronuclear DNA with a regular arrangement of restriction sites.

(v) Renaturation of Macronuclear DNA

(a) Rapid reassociation of DNA

Highly repeated sequences of DNA, for example the satellite DNA of higher eucaryotes, reassociate rapidly. Although both the CsCl density profile and the thermal melting profile suggest that the macronucleus lacks satellite DNA, it is nevertheless possible that there are present sequences which reassociate rapidly. In order to detect such sequences, macronuclear DNA was denatured and allowed to reassociate at 55°C in a spectrophotometer monitoring at 260nm. A slight drop in OD is observed indicating that some reassociation has taken place (Fig.15). Approximately 2% of the DNA reassociates under the conditions used, with a $C_0t_{\frac{1}{2}}$ of 2.1 moles sec l^{-1}. If this were treated as a single component and assuming that the GC content is similar to the bulk of the DNA, then the $C_0t_{\frac{1}{2}}$ can be corrected to 0.02 moles sec l^{-1}. This rate corresponds to a sequence complexity of 2.3×10^4 nucleotides and makes it unlikely that it is ribosomal DNA that is being assayed since the percentage of DNA involved is higher than could

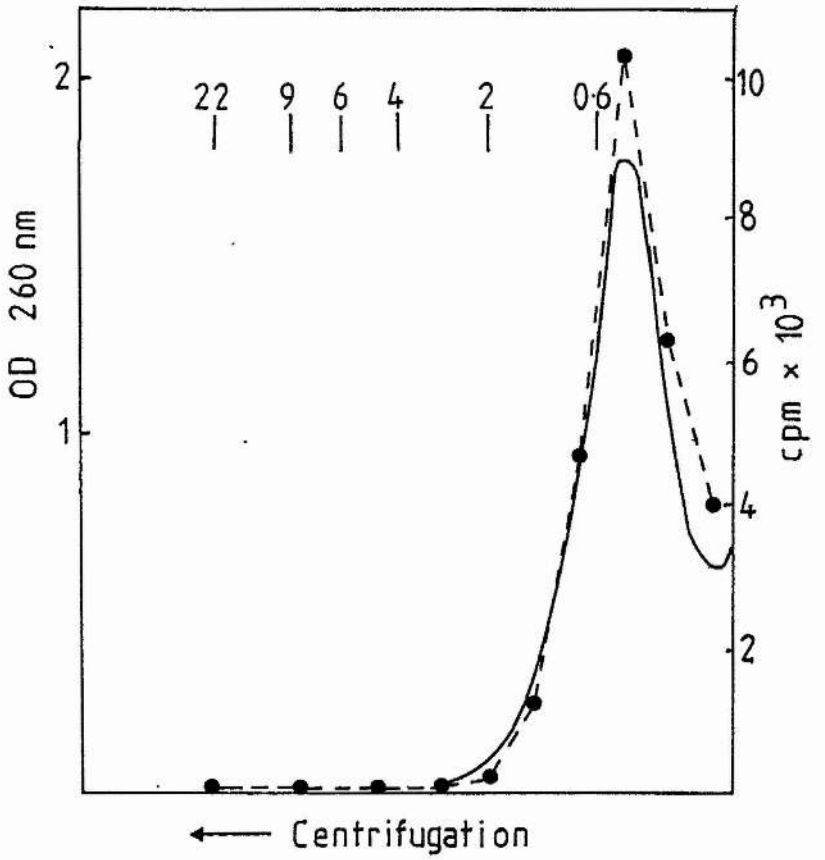

Fig. 17

Alkaline gradient centrifugation of sonicated and sonicated iodinated DNA.

Centrifugation condition used are described in the legend to Fig. 5.

●----● Iodinated sonicated DNA

——— sonicated DNA

be expected of ribosomal DNA, which should reassociate with a $C_o t_{\frac{1}{2}}$ of 20 times this value (Cummings, 1975).

(b) Optical renaturation of DNA

Needle-sheared samples of macronuclear DNA were denatured and allowed to renature for varying lengths of time. The extent of renaturation in each sample was measured by the percentage of the maximum hyperchromicity which had been reached.

Macronuclear DNA renatures simply (Fig. 16). The shape of the curve of renaturation is identical to that of E. coli indicating that there is very little if any repetitious DNA. The $C_o t_{\frac{1}{2}}$ is 110 moles sec l^{-1}. Since the DNA has a low GC content this value must be corrected in order to compare it with that of E. coli (Wetmur and Davidson, 1968). The correction factor is 0.52 assuming there to be a linear relationship between GC content and relative renaturation rates for DNA with a GC content as low as 25.5%. This may not be true and so the corrected value of $C_o t_{\frac{1}{2}}$ must be regarded as an approximate value.

For comparison a sample of E. coli DNA, was renatured under identical conditions. The $C_o t_{\frac{1}{2}}$ is 1.65 moles sec l^{-1}. This rate is lower than is normally found (e.g. Britten and Kohne, 1968). The lower value must be due largely to the fact that needle-shearing the DNA has not reduced the molecular length sufficiently. Samples of needle-sheared DNA were of variable size, ranging from 10S-20S on alkaline sucrose gradients, and of broad size range. Sonicated DNA was more

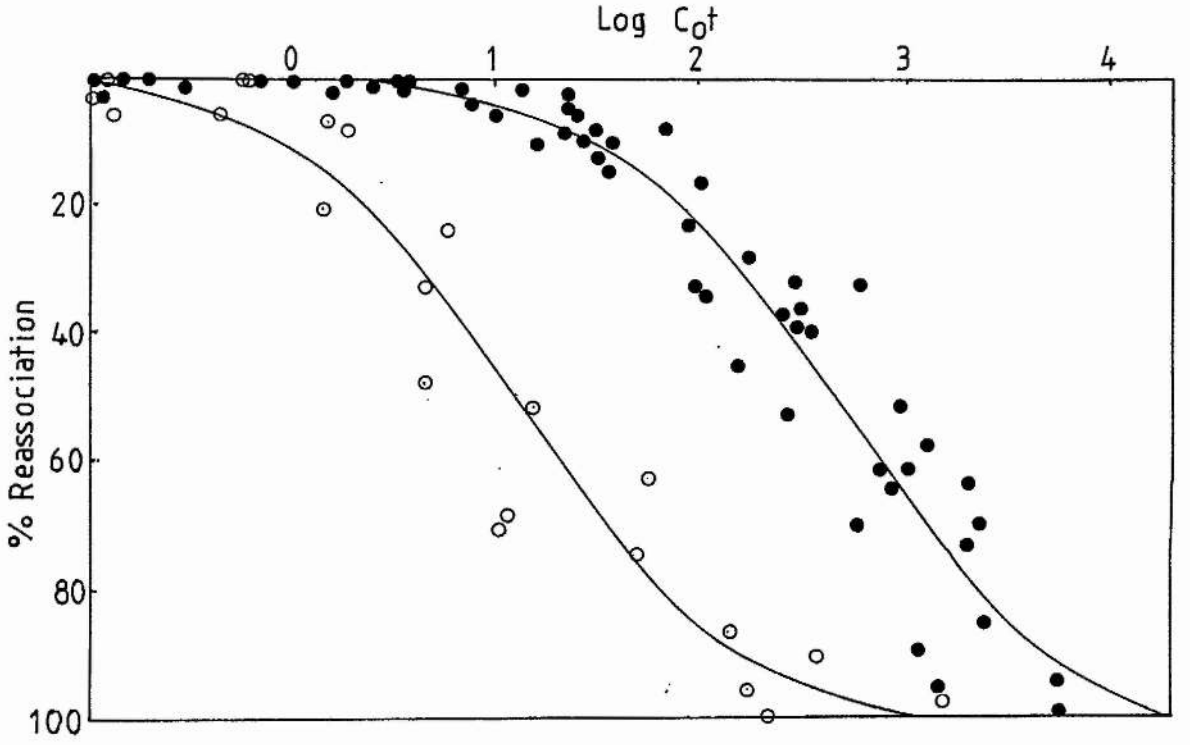

Fig. 18

Reassociation of Paramecium macronuclear DNA and E. coli.DNA. Each sample contained a small amount of homologous ^{125}I-labelled DNA. The percentage of reassociation was assayed by digestion with S_1-nuclease. Measurements obtained from several different experiments performed on different preparations are plotted on this graph.

● - Paramecia macronuclear DNA

○ - E. coli DNA

consistent having an average length of 400-500 base pairs (Fig. 1.7). For this reason sonicated DNA was used in future renaturation experiments. Optical renaturation also suffers from the disadvantage of the difficulty in measuring accurately the hyperchromicity of small samples of DNA. For this reason renaturation was carried out with I^{125} DNA as a probe. DNA could be iodinated to high specific activity and so the percentage of renaturation could be measured accurately in small samples.

(c) Renaturation of iodinated macronuclear DNA

Sonicated DNA was iodinated with I^{125} by the method of Commerford (1971). A small amount of iodinated DNA was added to each sample of native DNA in the ratio 1:1000. Samples were denatured and renatured for varying lengths of time and the extent of renaturation in each sample assayed by its resistance to single-strand specific S_1 nuclease. The results of several experiments are plotted on this graph (Fig. 18).

A parallel reassociation of iodinated E. coli DNA with an excess of unlabelled E. coli DNA was carried out under identical conditions, again for comparison. Here the $C_o t_{\frac{1}{2}}$ is 12mole sec. l^{-1}, higher than is normally found. This may be related to the use of iodinated DNA as the reaction probe (Axel et al.,1976). However the $C_o t_{\frac{1}{2}}$ value of 12mole sec. l^{-1} is much closer to the accepted value of 8mole sec. l^{-1} than is the value obtained by optical renaturation. It is assumed that any effect on rate of the

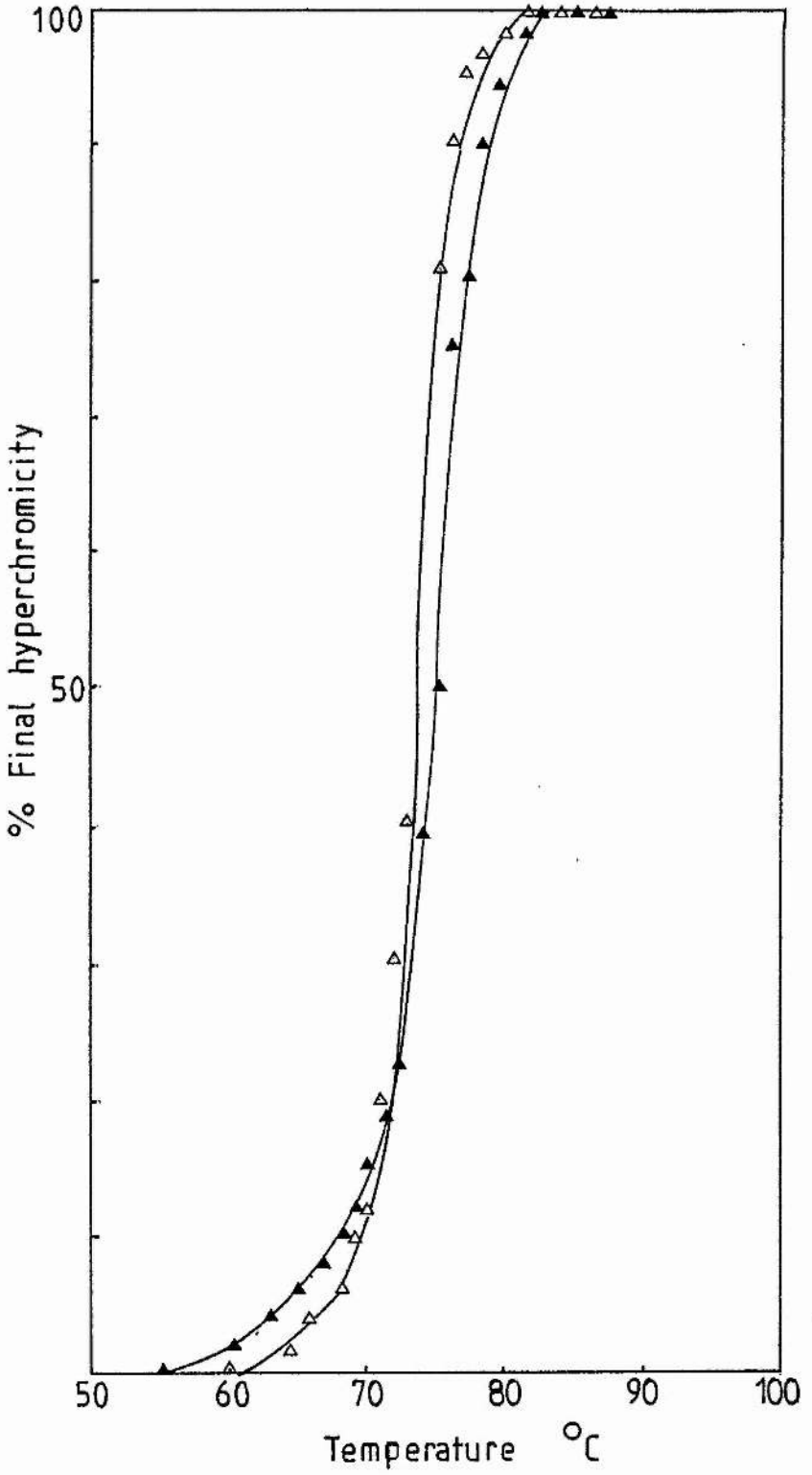

Fig. 19

Thermal dissociation of native and reassociated macronuclear DNA.

▲ - Native

△ - reassociated

use of an iodinated DNA probe is also found in the macronuclear DNA renaturation and that the renaturation kinetics and $C_o t_{\frac{1}{2}}$ values can be compared.

The renaturation kinetics of macronuclear DNA are again simple, with no evidence of the presence of repetitive DNA. The $C_o t_{\frac{1}{2}}$ of Paramecium macronuclear DNA is 450mole sec. l^{-1}. Correcting for the GC content gives a value of 230mole sec. l^{-1}. This is 19 times higher than the $C_o t_{\frac{1}{2}}$ of E. coli. Paramecium macronuclear DNA therefore has a DNA sequence complexity of 5.2×10^{10} daltons or 1.6×10^{8} nucleotides. The value of the genome size of E. coli is taken from Cairns (1963).

(d) Thermal stability of reassociated DNA

Reassociated DNA melts with a Tm just 1^{o}C below that of native DNA (Fig. 19). This means that the DNA is almost perfectly reassociated, supporting the observation that there is little or no repetitive DNA in the macronucleus. Any repetitive DNA would be expected to have diverged to some extent and, as such, would be responsible for a lowering of the Tm in a fraction of the DNA. The 2% of the DNA which appears to be repetitious is too small an amount to be detectable as a separate fraction in the melt of renatured DNA.

To determine if hybrids containing iodinated DNA were similarly well-matched, a sample of DNA which had been reassociated in the presence of iodinated DNA was melted from a column of hydroxyapatite. The Tm of 77.5-80^{o}C is slightly higher than that of native DNA, but this is charact-

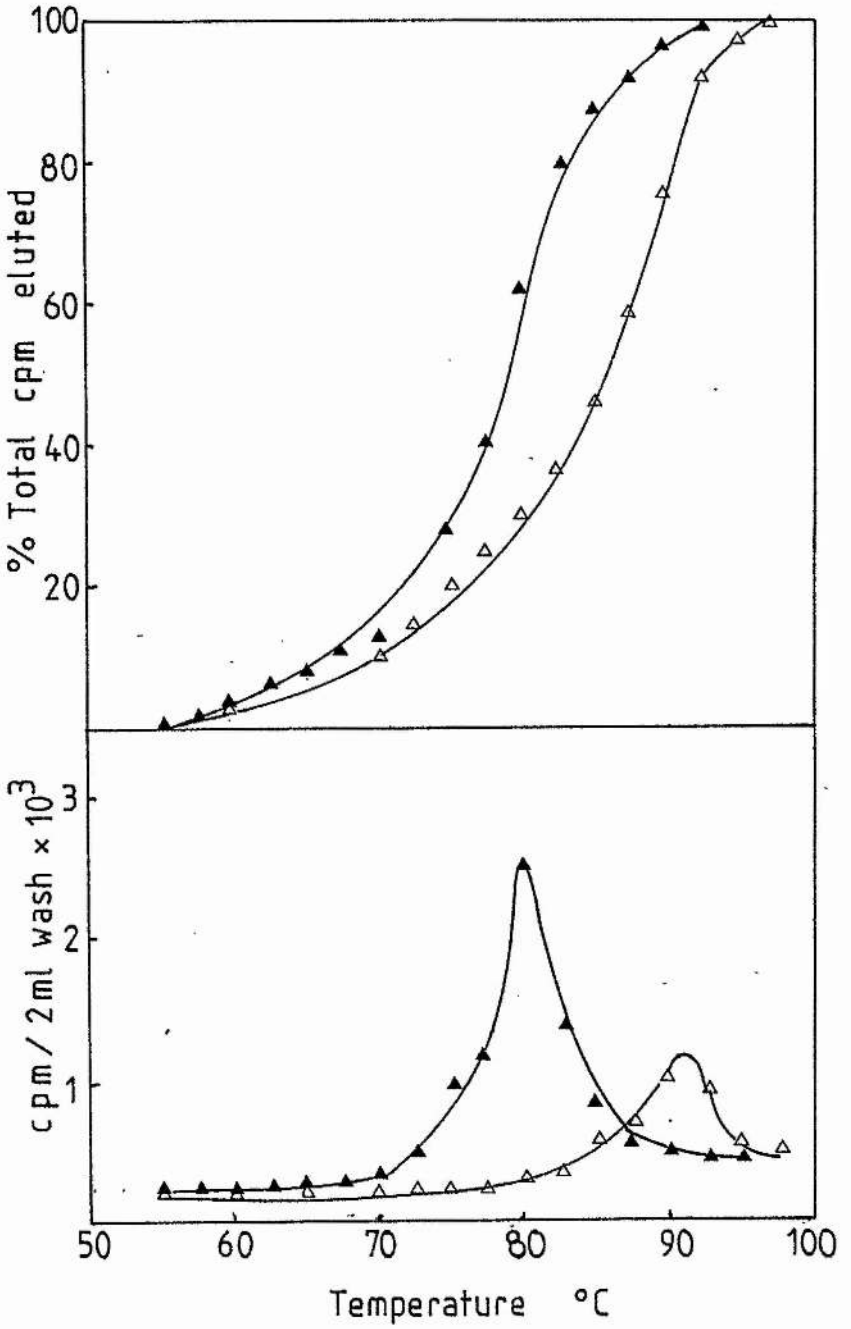

Fig. 20

Thermal dissociation of macronuclear DNA and E. coli DNA which had been reassoicated in the presence of homologous iodinated DNA.

Samples were applied to hydroxyapatite and the temperature raised in 2.5°C increments. Single stranded material was collected at each increment by washing the column with 2ml of pre-heated 0.12 M PB.

▲ Macronuclear DNA

△ E. coli DNA

istic of HAP melting as opposed to optical melting. Nevertheless this indicates that hybrids containing iodinated DNA are no less well-matched than normal hybrids. (Fig. 20).

4. Discussion

The DNA from macronuclei of Paramecium primaurelia stock 168 is simply organised. There are no density satellites and most, if not all, of the DNA has a GC content of approximately 25%. This is a low value in comparison with other organisms, but appears to be a general feature of ciliate macronuclear DNA (Allen and Gibson, 1971, Bostock and Prescott, 1972., Cummings, 1972., Pelvat and de Haller, 1976.)

Most of the macronuclear DNA renatures with very simple kinetics, indicating that there is little repetitive DNA. Only 2% of the DNA can be detected renaturing with a $C_o t_{\frac{1}{2}}$ approximately 200 times faster than the bulk of the DNA. Ribosomal DNA is known to be present as multiple copies in macronuclear DNA (Cummings, 1975). The 2% repetitive DNA should contain sequences of this type.

The remainder of the DNA renatures with a $C_o t_{\frac{1}{2}}$ of 450mole sec. l^{-1}. Because of the low GC content this must be corrected to 230mole sec. l^{-1} so that the value is comparable with that of E. coli. The corrected $C_o t_{\frac{1}{2}}$ value is 19 times higher than that of E. coli when renatured under identical conditions. The complexity of Paramecium macronuclear DNA thus corresponds to a complexity of approximately 5×10^{10} daltons or 1.6×10^{8} nucleotides.

The data obtained here with P. primaurelia stock 168 should be compared with the results of other authors. Most authors agree that macronuclear DNA has only one density component (Schildkraut et al.,1962., Allen and Gibson, 1971., Cummings, 1972) although Soldo and Godoy (1972), studying a stock of P. octaurelia, found a slight density shoulder on the heavy side of the main component.

Estimates of the GC content, as derived both from the bouyant density in CsCl and from the Tm value, vary according to author and to stock or species examined. However, the value for stock 168 of P. primaurelia of 25.5% GC falls within the range of 21-26% GC found by Cummings (1972) who was also studying stock 168 of the same species.

Estimates of DNA sequence complexity are somewhat variable. The value obtained in this work, 5×10^{10} daltons, is similar to the value of 3×10^{10} daltons for stock 513 of P. primaurelia (Cummings, 1975) and 3.8×10^{10} daltons for stock 299 of P. octaurelia (Soldo and Godoy, 1972). Both these sets of authors extracted DNA from the macronuclei. In contrast are the values obtained by Allen and Gibson (1972), for a variety of stocks and species, range from 1.84×10^{11} daltons in P. primaurelia to 2.6×10^{11} daltons in P. octaurelia These authors extracted DNA from whole cells. The evidence of culture conditions affecting DNA density values (Allen and Gibson, 1971) suggests contamination with bacterial DNA. This would also explain their observation that up to 15% of the DNA is repetitive. This "repetitive" DNA may in fact be bacterial DNA. Any contamination with bacterial DNA would

also increase the apparent $C_o t\frac{1}{2}$ value for macronuclear DNA giving an artifically high complexity value. However, even correcting for this possible effect, and also correcting for low GC content, the values obtained by Allen and Gibson (1972) are still higher than those obtained by Soldo and Godoy (1972), Cummings (1975) and in the present study.

Is there any sequence loss during macronuclear development in Paramecium? Unfortunately a direct comparison between macronuclear and micronuclear DNA cannot be made. However, the question of sequence loss can be indirectly assessed by first comparing the DNA sequence complexity of macronuclear DNA with the amount of DNA in the micronucleus. This is a measure of the number of macronuclear genomes which are present in the polyploid macronucleus. This value can in turn be compared with the number of micronuclear genomes in the macronucleus as determined by Woodward et al.(1966) who used the technique of comparative Feulgen micro-spectrophotometry. Sequence complexity reduction would be indicated by the former value being higher than the latter value.

Naturally, the calculation of sequence loss depends on an accurate measurement of the DNA content of the macronucleus. This has been measured in a number of ways, again with a wide disparity of results.

Since the sequence complexity of DNA from isolated macronuclei has been examined, the most relevant measurement for comparison is the DNA content of isolated macronuclei. Isolated from logarithmically growing cells, macronuclei contain on average 103pg of DNA. Since, in these conditions,

cells will contain nuclei at a whole range of DNA replication stages, the correction to G_1 nuclei gives a macronuclear DNA content of 70pg. (Cummings, 1972., Cummings and Tait, 1975., Skoczylas and Soldo, 1975).

Other less direct measurements of macronuclear DNA content give different values. For example Soldo *et al*. (1970) obtain the higher value of 103pg based on the DNA content of whole cells. This, however, depends on an accurate assesment of the cytoplasmic contribution. Behme and Berger (1970) obtained the value of 300pg based on the level of incorporation of tritiated thymidine from labelled *E. coli*, but they do not adequately account for the contribution of labelled mitochondrial DNA and ingested but undigested bacterial DNA. Gibson and Martin (1971) quote a value of 252pg of DNA based on photodensitometry.

Comparing the sequence complexity of 5×10^{10} daltons with the DNA content of 70pg, one obtains a ploidy value of 840, i.e. the G_1 macronucleus contains 840 copies of the macronuclear genome. Woodward *et al* (1966) showed that the macronucleus contains 860 copies of the micronuclear genome. In spite of the not insignificant errors involved in both these estimates, one can conclude that there is no gross sequence complexity reduction during macronuclear development in *Paramecium*.

Paramecium and *Tetrahymena*, both members of the holotrichous group of ciliates, are therefore quite distinct from the hypotrichs in terms of their macronuclear develop-

Organism	GC content	% Repetitive	Complexity[a]
Oxytricha[b]	42 %	0 %	13 ×
Stylonichia[c]	41 %	0 %	26 ×
Stentor[d]	32 %	15 %	20 ×
Tetrahymena[e]	30 %	10 %	13 ×
Paramecium[f]	26 %	2 %	19 ×

Table 1

Characteristics of macronuclear DNA from 5 species of ciliates.

a) Relative to the genetic complexity of E. coli.

b) From the data of Lauth et al, 1976.

c) From the data of Ammermann et al, 1974.

d) From the data of Pelvat and de Haller, 1976.

e) From the data of Borchsenius et al, 1978.

f) From the data in the text.

ment. The hypotrichs are members of the spirotrichous group of ciliates. The macronuclear DNA of another member of the spirotrichs has been examined, that of Stentor (Pelvat and de Haller, 1976). However, this appears to be more like the distantly related Paramecium and Tetrahymena than like the hypotrichs. Possibly gross sequence loss may be a feature unique to the hypotrichs. The fact that, of all the cilicates examined to date, only the hypotrichs show polytene chromosomes during macronuclear development strengthens the view that this type of development is unique.

If one examines ciliate macronuclear DNA, irrespective of the process of development, one finds that, among the limited number of ciliates examined, macronuclear DNA has very similar properties. (Table 1). Macronuclear DNA in the ciliates has low GC content, no satellite DNA and only small amounts, if any, of repetitive DNA. All calculated DNA sequence complexities lie within the range 13-20 times that of E. coli.

Naively, one could suggest that most ciliates require about the same number of genes - this being about $5\text{-}8 \times 10^4$ "average sized"(2000 base pair) genes , and that these genes are organised in a very similar fashion. The hypotrichs have, for some reason, a large amount of DNA in the micronucleus, some of which might have a function related to micronuclear activity or alternatively it may represent "junk" DNA which has been acquired during the course of evolution, as has been postulated for higher organisms. (Commoner, 1964, Ohno, 1972). Excess micronuclear DNA is

Organism	Species	Genetic complexity of unique component
CILIATE	Paramecium	$5{\cdot}2 \times 10^{10}$d
DINOFLAGELATE	Crypthecodinium cohnii [a]	$9{\cdot}8 \times 10^{11}$d
FUNGUS	Achyla ambisexualis [b]	$1{\cdot}4 \times 10^{10}$d
YEAST	Saccharomyces cerevisiae [c]	9×10^{9}d
SLIME MOULD	Dictyostelium discoideum [d]	$2{\cdot}8 \times 10^{10}$d
	Physarum polycephalum [e]	$3{\cdot}5 \times 10^{11}$d

Table 2

Genetic complexities of the unique component of the DNA of six species of eucaryote.

a) From the data of Allen et al. (1975)

b) From the data of Hudspeth et al. (1976)

c) From the data of Bicknell and Douglas, (1970)

d) From the data of Fonquet et al. (1974)

e) From the data of Firtel and Bonner (1972)

apparently unnecessary to macronuclear function and so is discarded during macronuclear development either by degradation or under-replication (Prescott et al., 1971). Other ciliates may also have excess micronuclear DNA which is removed during macronuclear development, but this may be a small amount in comparison with the bulk of "necessary" sequences.

If the ciliate macronuclear genome consists of necessary sequences, are they all transcribed? The ciliate macronucleus has 5-8 x 10^4 potential genes. This is within the range found in other primitive eucaryotes (Table 2). Yeast, for example, contains sufficient DNA to code for only 1 x 10^4 genes (Bicknell and Douglas, 1970) as has the fungus Achyla (Hudspeth et al., 1976). The cellular slime mould Dictyostelium discoideum could code for 5 x 10^4 mRNA species (Firtel and Banner, 1972), while the cellular slime mould Physarum polycephalum has a much higher genetic complexity and could code for up to 5 x 10^5 different mRNA species (Fouquet et al., 1974). The dinoflagellate Crypthecodinuim cohnii also has a high genetic complexity and could code for up to 1.5 x 10^6 mRNA species (Allen et al, 1975).

Higher eucaryotes generally have a higher genetic complexity than lower eucaryotes, but only a small percentage of the coding potential is realised as transcribed RNA. In mouse brain 20% of the genome is transcribed into RNA (Bantle and Hahn, 1976) but much lower values are found in other tissues. (Gelderman et al., 1971., Brown and Church, 1972., Grouse et al., 1972). The value of 20% of the mouse genome corresponds to 2-4 x 10^5 mRNA species. In considering the various developmental processes that are peculiar to multicellular organisms, one might expect that unicellular organisms should require fewer genes than, for instance

a mammal. Yeast apparantly trancribes only 4×10^3 genes (Hereford and Rosbash, 1977). In Achyla the value is lower, only 1×10^3 genes being transcribed (Timberlake et al.,1977) Dictyostelium however, transcribes many more genes, approximately 2.5×10^4 genes being transcribed, if one takes into account all stages of development (Firtel, 1972).

In terms of DNA sequence complexity, the ciliates are more similar to Dictyostelium than to yeast. In addition they are structurally more complex than yeast and display more complex behaviour patterns and so one might expect that they would transcribe possibly as many genes as are transcribed in Dictyostelium. This would constitute 25-35% of the ciliate genome.

Nothing is known of how much of the DNA is transcribed in the ciliates, except for the observation that 0.16% of Paramecium macronuclear DNA is transcribed into 25S ribosomal RNA and 0.28% into tRNA (Cummings, 1975). The question of how much of the DNA is transcribed into RNA is considered in the next chapter.

5. Summary

The macronuclear DNA of Paramecia primaurelia stock 168 is simply organised. It contains little repetitive DNA and has a sequence complexity 19 times higher than that of E. Coli. There is no evidence for a reduction in DNA sequence complexity during macronuclear development, in contrast to the situation in the hypotrichous group of ciliates.

The macronuclear DNA sequence complexity of *Paramecium* falls within the range found in other ciliates of 13-20 times that of *E. coli*. This is similar to values found in other lower eucaryotes.

CHAPTER III

Transcription of Macronuclear DNA

1. Introduction

Although an increasing amount is known about the organisation of the ciliate genome, little is known about its transcription. Such data as does exist on transcription has been gathered from work on only a few species of ciliates. It is evident from a comparative study of genome organisation among the ciliates that a particular feature observed in one ciliate is not necessarily present in another. This is probably also true of genome transcription. Nevertheless, in view of the scarcity of data on RNA structure and synthesis in the ciliates, it should perhaps be reviewed briefly as a background to the following work on the transcription of RNA in Paramecium.

Like almost all other cells, Paramecium contains large numbers of ribosomes. In size they are similar to eucaryotic ribosomes, having a sedimentation value of 80S. However, a closer examination shows that they differ in some ways from the ribosomes of higher eucaryotes (Reisner et al., 1968). For instance, in low magnesium concentration they dissociate into two subunits of 40S and 25S which are smaller than those of higher eucaryotes. Furthermore the ribosomal RNA, which has sedimentation values of 25.55 and 17.75, is intermediate in value between bacterial and higher eucaryotic ribosomes, as is the RNA: protein ratio of 54:46. Because of these differences Reisner et al., (1968) have described the ribosomes of Paramecium as primitive animal ribosomes.

Although most of the cellular RNA in Paramecium is ribosomal, the other eucaryote type of RNA can be found. PolyA$^+$ RNA can be isolated by oligo (dT)-cellulose chromatography (Hruby et al., 1977). Like higher eucaryotes, the polyA$^+$RNA of Paramecium is heterogenous in size and has a GC content similar to that of the DNA.

RNA is synthesised largely in the macronucleus. ^{14}C - uridine is rapidly incorporated into the macro nucleus and later appears in the cytoplasm (Kimball and Perdue, 1962). There is some evidence for RNA synthesis in the micro nucleus but this is difficult to determine with certainty and would, in any case, account for only a small proportion of the total RNA synthesis (Kimball and Prescott, 1964., Pasternak, 1967). It is claimed that RNA isolated from the Paramecium macronucleus consists largely of ribosomal RNA (Cummings, 1972). However, a 35S species of RNA is also found and, by analogy to higher eucaryotes, it is suggested that this is pre-ribosomal RNA.

RNA synthesis has been examined more fully in Tetrahymena (Prescott et al., 1971). Again a 35S species is found which gives rise to the 25 and 18S molecules. The 35S precursor is smaller than the 45S pre-ribosomal RNA found in higher eucaryotes, but is within the range found in other lower eucaryotes (Molgaard et al., 1976., Frankel et al., 1977., Planta et al., 1977). The small size of pre-ribosomal RNA in the lower eucaryotes suggests that processing in these organisms may be less extensive. In addition, processing of pre-ribosomal RNA Tetrahymena is very rapid, the 18S species appearing in the cytoplasm within 5 minutes of labelling. This may be a

consequence of the simpler nature of processing.

As well as the 35S ribosomal precursor, rapidly-labelled RNA in Tetrahymena contains a heterodisperse fraction ranging in size from 4-30S. This fraction turns over rapidly and probably represents messenger RNA. The size range is smaller than that of the rapidly labelled heterodisperse nuclear RNA of higher eucaryotes (cf Lewin, 1975). The observation that the size range of polyA$^+$ RNA in Paramecium is similar to that of the rapidly labelled heterodisperse RNA of Tetrahymena would suggest that, in the ciliates, mRNA is not transcribed as a high molecular weight precursor which requires processing befor
nsport into the cytoplasm. The absence of hnRNA in Tetrahymena
is also indicated by the work of Hermolin and Zimmerman (1976) who find no large rapidly labelled RNA in the nucleus.

In this respect RNA synthesis in the ciliates is similar to that of other lower eucaryotes. In Amoeba there appears to be no transcription of high molecular weight RNA (Prescott et al., 1971). Also, in a number of fungi and yeasts, there is little or no hnRNA formed (Fiala and Davies, 1965., Braun et al., 1966., Retel and Planta, 1967., Firtel and Lodish, 1973).

Although the RNA of ciliates appears to have some eucaryote features, there are clearly some differences, particularly with regard to its synthesis. This must be borne in mind when examining genome transcription in Paramecium. Transcription and control of the antigen genes can only be examined against the background of transcription of the rest of the genome. In

this Chapter, RNA synthesis, and the characteristics of the RNA formed, have been examined using a number of techniques. The transcription of the antigen genes and other genes associated with transformation are examined in more detail in Chapter IV.

2. Materials and Methods

(i) Preparation of RNA

(a) Preparation of polysomes

A washed pellet of cells at 0°C was mixed with 4 volumes of ice-cold homogenisation buffer (0.25M sucrose, 0.05M Tris (HCl), 0.025M KCl, 0.05M $MgCl_2$, 0.005M β-mercaptoethanol (β-SH), 150µg/ml polyvinyl sulphate (PVS) or dextran sulphate, 100µg/1ml heparin pH 7.6). The cells were homogenised using a teflon/glass homogeniser by 20 strokes at speed 8 in a Tri-R stirrer. The homogenate was centrifuged at 500xg for 10 minutes at 4°C to remove large debris and the supernatant centrifuged at 10,000 x g for 10 minutes to pellet mitochondria and bacteria. This post-mitochondrial supernatant (PMS) was used in several subsequent preparations. The polysome profile of the PMS was examined by layering 0.2 ml on a 12 ml 15-30% sucrose gradient made up in 50 mM Tris (HCl), 25 mM KCl, 5 mM $MgCl_2$, 5 mM β-SH pH 7.6 with a 2ml 60% sucrose pad made up in the same buffer. The gradient was centrifuged at 27,000 rpm for 2 hours at 4°C in a 6 x 14 ml MSE titanium swing-out rotor in a MSE Superspeed 65 centrifuge. At the end of this time the gradient tube was pierced and the gradient pumped through a flow cell recording at 260 nm. The profile was recorded on a chart-recorder.

A preparative amount of polysomes was made by layering 3 ml of the PMS on a 35 ml 15-30% sucrose gradient with a 5 ml 60% sucrose pad made up in the same buffer as above. This gradient was centrifuged at 23,000 rpm in a 6 x 38 ml titanium swing-out rotor at 4°C for 3 hours. After this time the gradient tube was pierced and the gradient pumped through a flow cell

recording at 260 nm. Appropriate fractions were collected in a Gilson fraction collector. The polysomes were pelleted from the collected fractions by centrifuging at 120,000 x g for 2 hours.

A crude preparation of polysomes could be made directly from the PMS by centrifuging the PMS at 120,000 x g for 1 hour and recovering the pellet. Polysomes could be removed from membranes by treating the PMS with a mixture of 0.5% Nonidet P40 and 0.5% sodium deoxycholate.

(b) Preparation of RNA from PMS and polysomes

The PMS was adjusted to 50mM Na_2EDTA to dissociate ribosomes and then adjusted to 0.5% sarkosyl to dissociate protein from RNA. The mixture was adjusted to 0.1M NaCl and phenol extracted as described previously. The aqueous phase was extracted once more with phenol mixed in a ratio of 1:1 with chloroform and then extracted with chloroform alone. The aqueous phase was adjusted to 0.1M sodium acetate buffer pH 5.2 and the nucleic acid precipitated by the addition of 2 volumes of absolute alcohol. The nucleic acid was allowed to precipitate overnight at -20°C before being recovered by centrifugation at 2,000 x g for 20 minutes at 4°C.

RNA was extracted from pelleted polysomes by raising the pellet in homogenisation buffer and treating it as described above.

(c) Preparation of RNA from intact cells

RNA was prepared from intact cells by a modification of the method used by Hruby et al., (1977). A washed pellet of cells at 0°C was mixed with 2 volumes of Kirbys buffer

(1% Tri-isopropylnaphthalene sulphonate, 6% sodium 4-amino-salicylate, 30 mM NaCl, 50 mM Tris (HCl), 10 mM EDTA, 6% v/v phenol pH 7.8). To this was added one tenth volume of 1M glycine buffer pH 9.5 and one fifth volume of 5% sodium deoxycholate pH 9.5. The mixture was adjusted to 0.1% diethylpyrocarbonate and homogenised in a teflon/glass homogeniser by 20 strokes at speed 8. An equal volume of phenol was added and the mixture stirred at room temperature for 15 minutes before being centrifuged to separate the phases. The aqueous phase was reextracted with phenol/chloroform and again with chloroform before being adjusted to 0.1M sodium acetate buffer pH 5.2 and precipitated with 2 volumes of absolute alcohol. The nucleic acid was allowed to precipitate overnight at -20°C and recovered by centrifugation for 30 minutes at 4°C .

(d) Preparation of RNA from macronuclei

Macronuclei were prepared as described in Chapter II, Materials and Methods. The pellet of nuclei was taken up in Kirbys buffer, then glycine buffer, deoxycholate and diethyl pyrocarbonate were added and the mixture homogenised as described above. The remainder of the RNA extraction procedure was as described above.

(e) Preparation of RNA from reticulocytes

Dutch rabbits, 2-3 kg, of either sex, were made anaemic by 5 daily injections of 15mg acetyl phenyl hydrazine in rabbit saline (0.13M NaCl, 7.5mM $MgCl_2$, 5mM KCl) (Darnborough et al., 1973). On the first day an additional injection of 10mg vitamin B_{12} and 100mg folic acid was given. 10 ml of blood was removed from the marginal ear vien 9-10 days after the

first injection. The blood was collected in rabbit saline containing 0.1% heparin and the blood cells pelleted by 5 minutes centrifugation at 2,000g. The cells were washed twice with 5 volumes of rabbit saline and the white cells removed from the surface of the pellet. The cells were lysed by the addition of an equal volume of ice cold water. The lysate was centrifuged at 27,000g for 15 minutes to remove cell debris and an equal volume of Kirbys buffer added to the supernatant. Glycine buffer, deoxycholate and diethyl pyrocarbonate were added and the mixture phenol extracted as described before.

(f) Isolation of Poly A^+ RNA

The alcohol precipitated nucleic acid from any of the above preparations was centrifuged at 2,000g for 20 minutes at 4°C. The supernatant was removed and the pellet dried *in vacuo*. It was raised in 1-5ml 'DNAse' buffer (10 mM Tris (HCl), 10 mM NaCl, 10 mM $MgCl_2$ pH 7.4) and 10µg deoxyribonuclease (Sigma electrophoretically pure) added to each ml of solution. It was digested at 37°C for 30 minutes and the reaction stopped by the addition of 10mM EDTA, 0.5% SDS. 50µg proteinase K (Boehringer) was added and digestion continued at 37°C for a further 30 minutes. Residual protein was removed by the addition of one tenth volume of 1M sodium acetate buffer pH 5.2 and 2 volumes of absolute alcohol.

After overnight precipitation at -20°C the RNA was pelleted by centrifugation at 2,000g for 20 minutes, the pellet dried and taken up in 5ml oligo(dT)-cellulose binding buffer (0.4M NaCl, 10mM Tris HCl, 1mM EDTA, 0.2% SDS pH 7.8). This was applied slowly to a column containing 0.25g

oligo-(dT)-cellulose (Boehringer) which had been washed with the same buffer. The eluate was passed through a flow cell recording at 260nm, connected to a chart recroder. When the sample had passed through the column, it was washed with binding buffer until the OD returned to the base-line. The column was then washed with 5 ml of wash buffer (10mM Tris HCl, 1mM EDTA, 0.2% SDS, pH 7.8). Poly A^+ RNA previously bound to the column was eluted by this buffer and seen as a peak on the chart recorder. The peak was collected, adjusted to 0.1M sodium acetate pH 5.2 and precipitated with 2 volumes of alcohol. After overnight precipitation, the RNA was pelleted and re-chromatographed on the oligo(dT)-cellulose column as before.

(ii) Analysis of RNA

(a) Aqueous non-denaturing gradients of RNA

Samples of RNA were routinely examined on a 15-30% NETS gradient. This was a linear gradient of 15-30% sucrose made up in 0.1M NaCl, 0.1M Tris HCl, 10mM EDTA, 0.2% SDS, pH 7.6. Samples were dissolved in 0.1-0.2ml of the same buffer and overlaid on a 12ml NETS gradient. This was centrifuged at 22,000 rpm for 20 hours at 20°C. The gradient tube was pierced and the gradient pumped through a flow-cell recording at 260nm. The profile recorded on a chart-recorder. For some purposes 0.5-1ml fractions were collected with a Gilson fraction collector.

(b) Hybridisation of gradient fractions to tritiated poly (U)

1ml fractions from a NETS gradient were precipitated

with alcohol in the presence of 50µg of yeast or E. Coli tRNA as carrier. After overnight precipitation at -20°C, the samples were centrifuged, the pellet rinsed with 0.5ml 70% alcohol and dried in vacuo before being taken up in 0.5ml SSC. To each sample was added 0.05mCi (20-74 Ci/mole) ^{3}H-poly (U) (Radiochemical centre, Amersham) and samples were incubated at 45°C for 30 minutes and then chilled. 0.5ml of 25µg/ml RNAse A was added to each sample and incubated at 0°C for 30 minutes. 1ml 20% TCA was added, and, after 30 minutes at 0°C, samples were filtered through 2.5cm GFA filters. The filters were washed with 10ml of 5% TCA and 5ml of 96% alcohol before being dried and counted in 5ml of toluene-based scintillant NE233 (Nuclear Enterprises, Edinburgh).

(c) Sizing of poly (A) tract

Poly A^{+} RNA was prepared from cells which had been labelled with tritiated adenosine (see Section iii a). The poly A^{+} RNA was dissolved in 1ml 0.1M NaCl, 0.01 M EDTA, 0.01M Tris HCl pH 7.4 at a concentration of 0.1-0.2 mg/ml. 2 µg pancreatic ribonuclease A and 10 units of ribonuclease T_1 were added and the sample incubated for 30 minutes at 37°C. The sample was then placed on ice and, when cold, 100µg of E. Coli tRNA was added and the digest precipitated with 2 volumes of absolute alcohol.

The poly(A) tract was analysed in a 15% acrylamide 10cm x 0.5 cm diameter disc gel. This was basically a Loening gel (Loening, 1969) containing 0.2% SDS. The sample was centrifuged at 2,000g for 20 minutes, the pellet dried and taken up in 50µl

gel buffer containing 0.1% bromophenol blue and 20% glycerol. 50µl of a solution containing 100µg 4S and 5S RNA (derived from the 40S storage particles of Triturus cristatus carnifex oocytes) was added to act as markers. The gel was electrophoresed at 10V/cm for 2-3hours, scanned in a Joyce/Loebel scanner and sliced into 2mm sections. Each slice was eluted with 1ml of hydrogen peroxide at 70°C for 24hrs and counted in 5ml of dioxan-based scintillation cocktail NE 250 (Nuclear Enterprises, Edinburgh).

(d) Hybridisation of poly A^+RNA with iodinated ^{125}I-DNA

Poly A^+ RNA was dissolved at a concentration of 1mg/ml in 0.5M NaCl, 10mM Pipes pH 6.5 in the presence of a small amount of ^{125}I-DNA. 25µl aliquots were sealed in 50µl capillary tubes and treated at 100°C for 5 minutes. They were transferred to a 55°C waterbath and samples removed to ice at time intervals. The extent of hybridisation in each sample was assayed by S1 nuclease digestion of each sample as described previously in Chapter II Materials and Methods. A sample taken from the 100°C waterbath and placed immediately in ice acted as the zero time sample. This value was subtracted from sample values.

(e) Preparation of cDNA

cDNA was prepared by the method of Bishop et al., (1974) AMV reverse transcriptase was obtained from the Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda. For assay of the enzyme, tritiated deoxy-TTP at a specific activity of 43 Ci/mmole was used with a Poly (A) template.

For reverse transcription of poly A^+ RNA a mixture of tritiated deoxy TTP and deoxy CTP at a specific activity of 21 Ci/mmole was used. The reaction was incubated for 1hour at 42°C then treated with alkali for 2 hours, neutralised, phenol extracted and applied to a 50 cm x 0.5 cm diameter (10ml) column of G50-Sephadex equilibrated in 0.3M NaCl. 2ml was collected in the void volume and the cDNA precipitated with 2 volumes of alcohol in the presence of 50µg of E. Coli tRNA. A sample of the cDNA was routinely sized on a 12ml alkaline sucrose gradient (5-20% sucrose in 0.9M NaCl, 0.1M NaOH). The gradient was centrifuged at 25,000 rpm for 15 hours at 10°C and compared with an identical gradient run under the same conditions which had been overlaid with marker DNA (HindIII digested λ DNA, Boehringer).

(f) cDNA-DNA hybridisation

Sonicated macronuclear DNA at approximately 1mg/ml in 0.5M NaCl, 10mM Pipes was mixed with a trace amount of cDNA. 25µl aliquots were sealed in 50µl capillary tubes and heated for 5 minutes in a 100°C water-bath before being transferred to a water-bath at 55°C. Samples were removed at intervals and the percentage of the cDNA which had been made double-stranded assayed by S1 digestion of the sample. The samples were then TCA precipitated with 100µg of BSA as a carrier, filtered through glass-fibre filters, washed with TCA and alcohol, dried and counted in 5ml toluene based scintillation cocktail (NE233) (Nuclear Enterprises, Edinburgh).

(g) cDNA- poly A^+ RNA hybridisation

Paramecium poly A^+ RNA was dissolved at concentrations of 100µg/ml and 1mg/ml in 0.5M NaCl, 10mM Pipes pH 6.5. A small

amount of cDNA was added to each concentration and treated as described above.

Reticulocyte poly A^+RNA was dissolved in 0.5M NaCl, 10mM Pipes pH 6.8 at a concentration of 1 and 10µg/ml with 100µg/ml E. coli tRNA. After this, the samples were treated as described above. A sample containing cDNA alone was digested with S_1 nuclease to determine the extent of S_1 digestibility of the cDNA. This value, usually 5-10%, was subtracted from all sample values.

(h) Thermal melting profile of cDNA-poly A^+RNA hybrids

A 5µl sample of cDNA-poly A^+ RNA was hybridised at 55°C to a Rot value of 1000 and was then dissolved in 2.5 ml of 0.1 SSC at 50°C. This was placed in a thermostatically controlled water-bath and the temperature raised in 5°C increments. At each temperature increment the sample was allowed to equilibrate and a 0.2 ml sample removed and placed on ice. When all samples had been taken, the extent of cDNA remaining double-stranded was assayed by S_1 nuclease digestions.

(iii) Labelling of RNA with radioisotopically labelled nucleotides.

(a) Labelling of paramecia using labelled bacteria

Bacteria were grown in a small volume (20-50ml) of limiting medium (0.5 mg/ml Na_3 citrate $5H_2O$, 0.1 mg/ml $MgSO_4$ $7H_2O$, 1.0 mg/ml $(NH_4)_2SO_4$, 7.0 mg/ml K_2HPO_4, 2 mg/ml KH_2PO_4, 1mg/ml glucose). This allowed the bacteria to grow to 1mg/ml wet weight. The medium was sterilised by autoclaving, innoculated with Klebsiella aerogenes and incubated in a 37°C waterbath with air bubbling through the medium. When sufficient growth had

occurred to make the medium slightly cloudy, the labelled material was added. This was either 0.1 -1.0 mCi tritiated nucleotide (20-27 Ci/ mmole) for labelling nucleic acids or 0.1-1.0 mCi ^{35}S-labelled magnesium sulphate for labelling protein. Growth was continued overnight and the bacteria harvested by centrifuging the culture at 10,000 x g for 10 minutes. The pellet was rinsed and resuspended in 1-2 ml MS.

Paramecia were harvested as described previously and washed twice with 100ml of MS to remove exogenous bacteria and resuspended in MS as a 0.5-1% suspension (0.5-1ml packed cells in 100ml). Approximately 0.1 mg/ml labelled bacteria were added and the cells incubated with the bacteria for varying lengths of time before being washed and either harvested immediately or placed in grass medium containing unlabelled bacteria.

For long-term labelling with bacteria, 100ml of cleared paramecia culture was added to 1litre sterile grass medium together with labelled bacteria from 50ml of limiting medium. The culture was allowed to undergo 3 fissions before being harvested.

(b) Radioisotopic labelling of paramecia in the absence of bacteria

Paramecia were harvested, washed twice in MS and resuspended as a 0.5% culture in MS containing 10mM sodium formate. The cells were incubated in this for 15-30 minutes to eat and partially digest any remaining bacteria. 0.1 mCi of tritiated uridine (27 mCi/ mmole) was added and the cells incubated for varying lengths of time. 15 mg/ml cold uridine was added at the end of this time and the cells either harvested

immediately or left for a further period of incubation before harvesting. This is a modification of the method used by Cummings (1975).

(c) Labelling of paramecia in the presence of inhibitors

Cells were labelled as described above (a) or (b), with the addition of 0.2-5 µg/ml actinomycin D or 1-5 µg/ml ∝ amanitin

(iv) Cell Free Synthesis

(a) Wheatgerm in vitro synthesis

Wheatgerm extract was prepared as described by Roberts and Patterson (1973). Incorporation was assayed under the following condition:- 20µl wheatgerm extract, 20 mM Hepes buffer, 2 mM dithiothreitol, 1 mM ATP, 20 µM GTP, 8 mM creatine phosphate, 40 µg/ml creatine kinase, 20-30 µM unlabelled aminoacids, 80 µM KCl, 3 mM magnesium acetate, 5 µl ^{35}S - methionine (57 Ci/ mmole), 2 µg poly A^+ RNA. The reaction mix was incubated at 25°C and 5µl samples taken at various times up to 90 minutes. Samples were diluted into 1 ml 1 mg/ml cold methionine, 0.5 ml of 1N NaOH added and incubated for 15 minutes at 37°C. 100 µg of BSA and an equal volume of cold 10% TCA were added. The samples were vortex-mixed, placed on ice for 30 minutes and filtered through 2.5 cm GFA filters which were dried and counted in toluene-based scintillation cocktail.

(b) Reticulocyte lysate

Rabbits were made anemic by the method described previously for the preparation of reticulocyte poly A^+ RNA. The reticulocyte lysate was prepared according to Hunt et al. (1972). Washed blood cells were lysed by the addition of 5 volumes

of ice cold 5 mM $MgCl_2$. After 1 minute the lysate was made isotonic by the addition of 0.75 volumes of 2M sucrose. Cell debris was removed by centrifugation at 12,000 x g for 15 minutes. The clear supernatant was decanted and stored at -80°C.

The lysate was incubated and assayed according to the method of Pelham and Jackson (1976) although usually 80µl reaction mixtures were used to test incorporation rather than 0.8 ml. The radioactive amino acids used were either ^{35}S-methionine (57 Ci/ m mole) or ^{14}C amino acids (57 mCi/ mAtom) both from Radiochemical Centre, Amersham.

Commercially prepared reticulocyte lysates obtained from New England Nuclear and Radiochemical Centre, Amersham were also tested using Paramecium poly A^+RNA as template.

(c) Analysis of translation products

To 10µl samples was added 5µl gel sample buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, 0.1M dithiothreitol, 0.0625M Tris HCl pH 6.8). Samples were boiled for 3-5 minutes, cooled and applied to a 1mm 15% acrylamide slab gel with a 5% staking gel. The electropheresis buffer was (0.025 M Tris HCl, 0.192 M glycine, 0.1% SDS, pH 8.3). The gel was electrophoresed at 100V for 20 hours, stained for 30 minutes in 40% Methanol, 10% acetic acid 0.1% Coomassie-brilliant blue and destained in 10% methanol. 10% acetic acid. The destained gel was dried down onto filter paper and exposed to autoradiographic film (Fuji X-ray film, Fuji Photo Film Co.) for 1-2 weeks before being developed in Phenisol developer (Ilford).

(v) Visualisation of Transcription units

(Modified from Miller and co-workers; Miller and Beatty (1969) and Miller and Bakken (1972)).

Paramecia were harvested and washed with MS. A 2µl droplet containing about 20-30 cells was added to a 5µl drop of sucrose - "Joy" solution (0.05M Sucrose, 0.1% "Joy" (Proctor and Gamble), 100 µg/ml yeast RNA type X1, 100 µg/ml heparin, adjusted to pH 8.7 with borate buffer pH 9.2). This mixture was left 10-15 minutes until almost all the cells had been lysed. A 15 µl droplet of pH 9 water (distilled water adjusted to pH 9.0 with borate buffer) was added and the cell contents allowed to disperse for a further 30 minutes.

Copper electron microscope grids Ve-Co 300 mesh coated with a thin carbon film were glow-discharged for 1-5 minutes at 100-150 mTorr using a Balzers Micro-BA3 machine. Discharged grids were rinsed in 96% alcohol and in sucrose/paraformaldehyde (0.1 M Sucrose, 10% paraformaldehyde adjusted to pH 8.5 with 0.1M NaOH). They were then placed in a centrifuge chamber containing 50µl of sucrose/paraformaldehyde. The dispersed cells were layered over the sucrose/paraformaldehyde cushion and centrifuged at 3500 rpm for 5-10 minutes. The grids were then removed from the chambers, rinsed for 10 seconds in 0.4% Photoflo (Kodak) which was adjusted to pH 8.6 with borate buffer, and air dried. The grids were stained for 1-5 minutes in 1% PTA in 70% alcohol, rinsed for 15 seconds in each of 96% alcohol, 100% alcohol, isopentane and air dried. The grids were rotary shadowed with Pt/Pd (80:20) at an angle of 8° using a Balzers Micro BA-3. They were examined in a Philips-301 electron microscope.

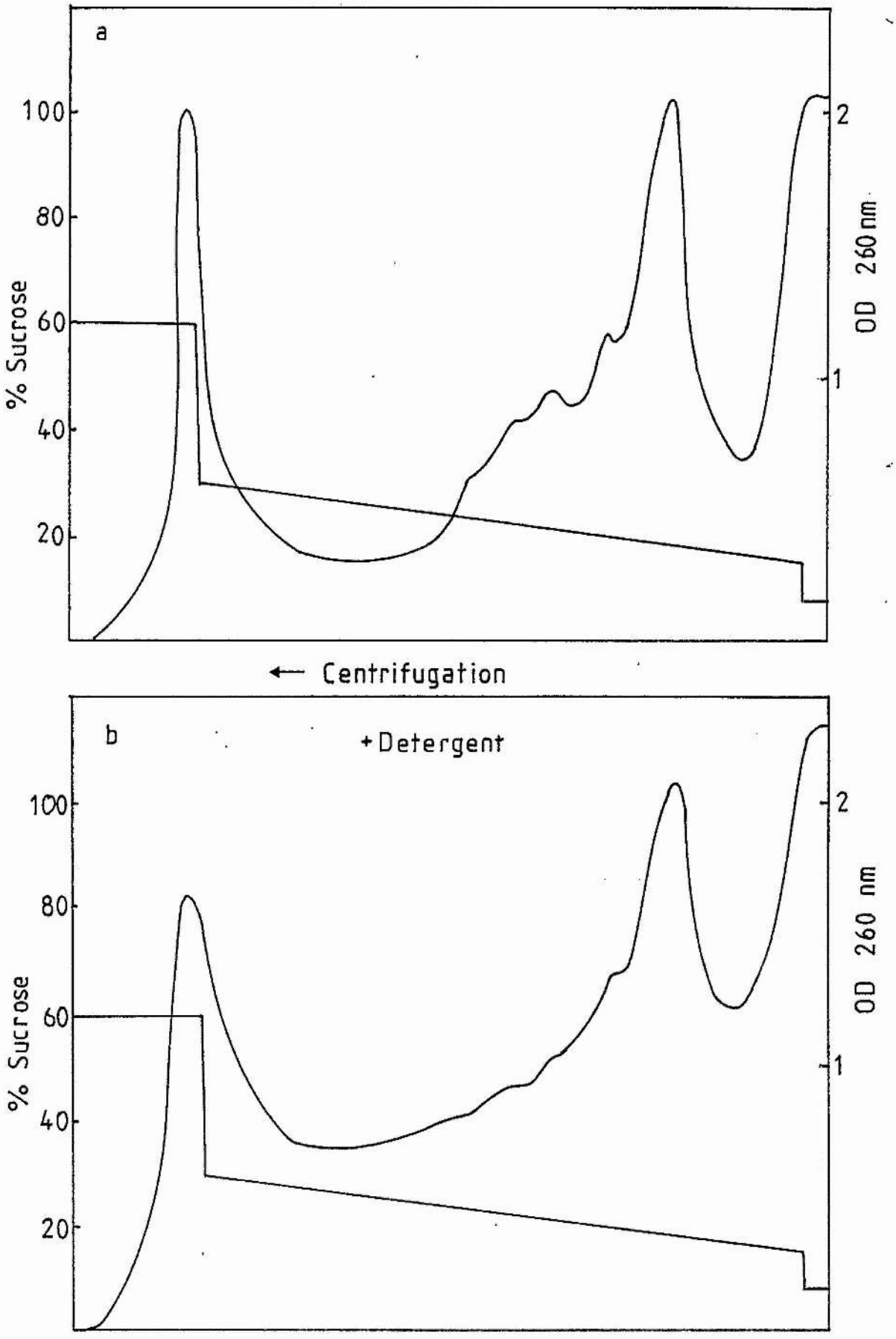

Fig 21 a and b

Optical profiles of two samples of PMS centrifuged on a sucrose gradient.

a) Untreated PMS
b) Detergent treated PMS

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3. Results

(i) Isolation and Characterisation of RNA

(a) Polysomes in paramecia

RNA was initially prepared from the "post-mitochondrial supernatant" (PMS). To prepare this, cells were homogenised in a buffer containing K^+, Mg^{++}, mercaptoethanol, Tris HCl and sucrose, furthermore a buffer in which ribosomal and polysomal structure would be preserved. Dextran sulphate and heparin were included to inhibit RNAse activity. The homogenate was subjected to two centrifugation steps. The first was a low speed spin to remove intact cells, large cell debris and nuclei, and the second a higher speed spin to remove cell particles the size of mitochondria, bacteria and nuclear fragments.

A sample of this supernatant, centrifuged on a sucrose gradient, shows a characteristic profile which can be divided into 4 zones (Fig. 21a). A considerable proportion of the material absorbing at 260nm pellets onto, and through, the dense sucrose interphase. This fraction has previously been examined chemically and by electronmicroscopy (Sommerville and Sinden, 1968) and contains membrane bound ribosomes. This can also be demonstrated by treating the PMS with nonionic detergents, resulting in a decrease in the size of the interphase peak and an increase in the amount of free polysomes (Fig 21b).

The main peak of the gradient corresponds to single ribosomes (Sommerville and Sinden, 1968). The area between the membrane bound peak and the monosome peak shows the characteristic shoulders, or peaks, of various size classes of polysomes. Polysomes consisting of up to six ribosomes can be distinguished. Clearly there are polysomes larger than this, but the individual

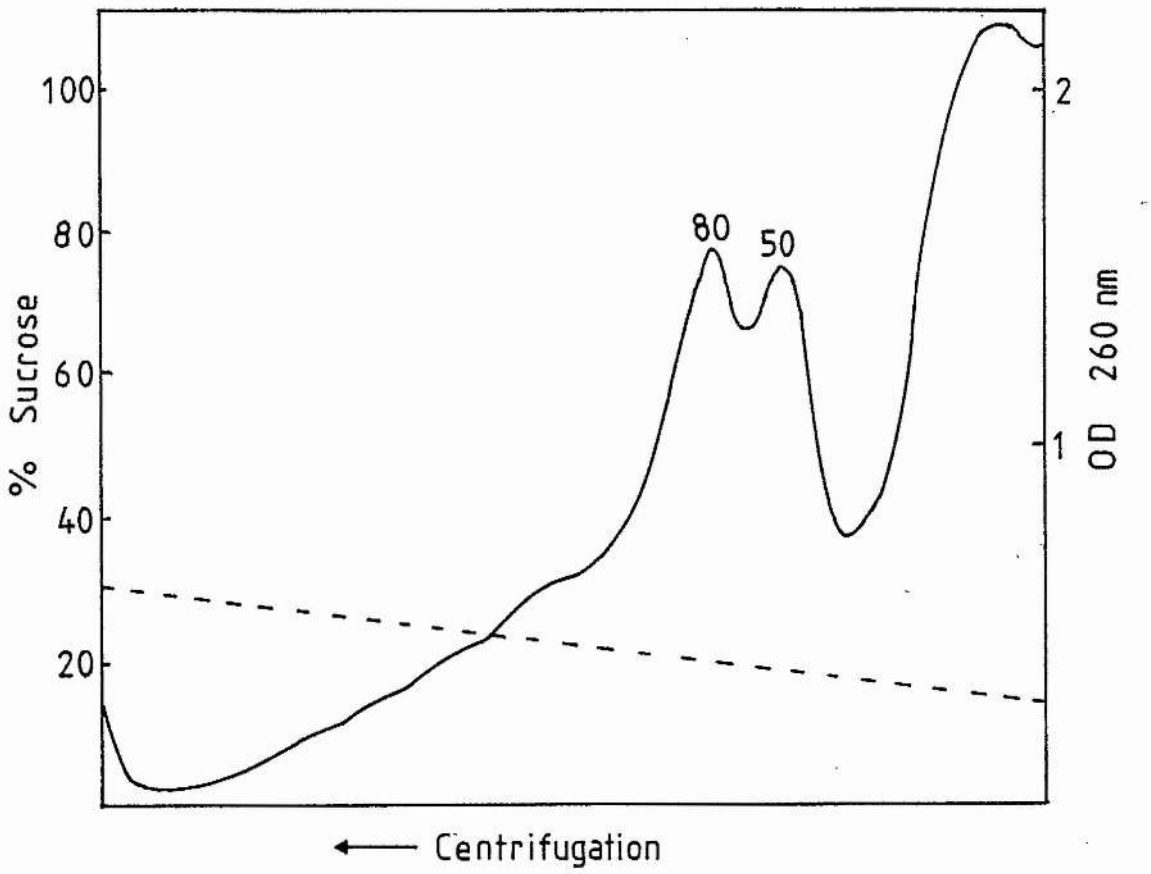

Fig. 22

Optical profile of a sample of PMS centrifuged on a 15-30% sucrose gradient.

The numbers indicate the S-value of the two peaks.

size-classes are not resolved. Optically absorbing material is observed as large as 290S which would correspond to about 30 ribosomes (cf Sinden, 1973). Any polysomes larger than this would be expected to pellet into the dense sucrose interphase.

Both the ratio of polysomes to monosomes, and the range of polysome sizes, varies from preparation to preparation. This variation is also observed by Reisner and Bucholtz (1972) and may be due to varying levels of endogenous ribonuclease activity. This, in turn, may be related to the growth stage of the culture. As mentioned in Chapter II, Cummings (1975) finds a variation in DNA size which depends on the growth stage of the culture. Again this may be due to variation in endogenous nuclease activity.

In some preparations, a shoulder or peak on the light side of the monosome peak can be seen (Fig. 22). This was associated with a low amount of polysomal material. This peak has a sedimentation value of approximately 50S and probably corresponds to the 60S hydrated form of the ribosome described by Reisner et al. (1968).

(b) RNA from the post-mitochondrial supernatant

In order to examine the types of RNA present in the PMS, it was treated with EDTA and SDS to disrupt polysomes and ribosomes and subsequently phenol-extracted to remove protein. The RNA was precipitated and redissolved in NETS buffer (NaCl, EDTA, Tris HCl, SDS). It was examined by centrifugation on a 15-30% nondenaturing sucrose gradient made up in the same buffer.

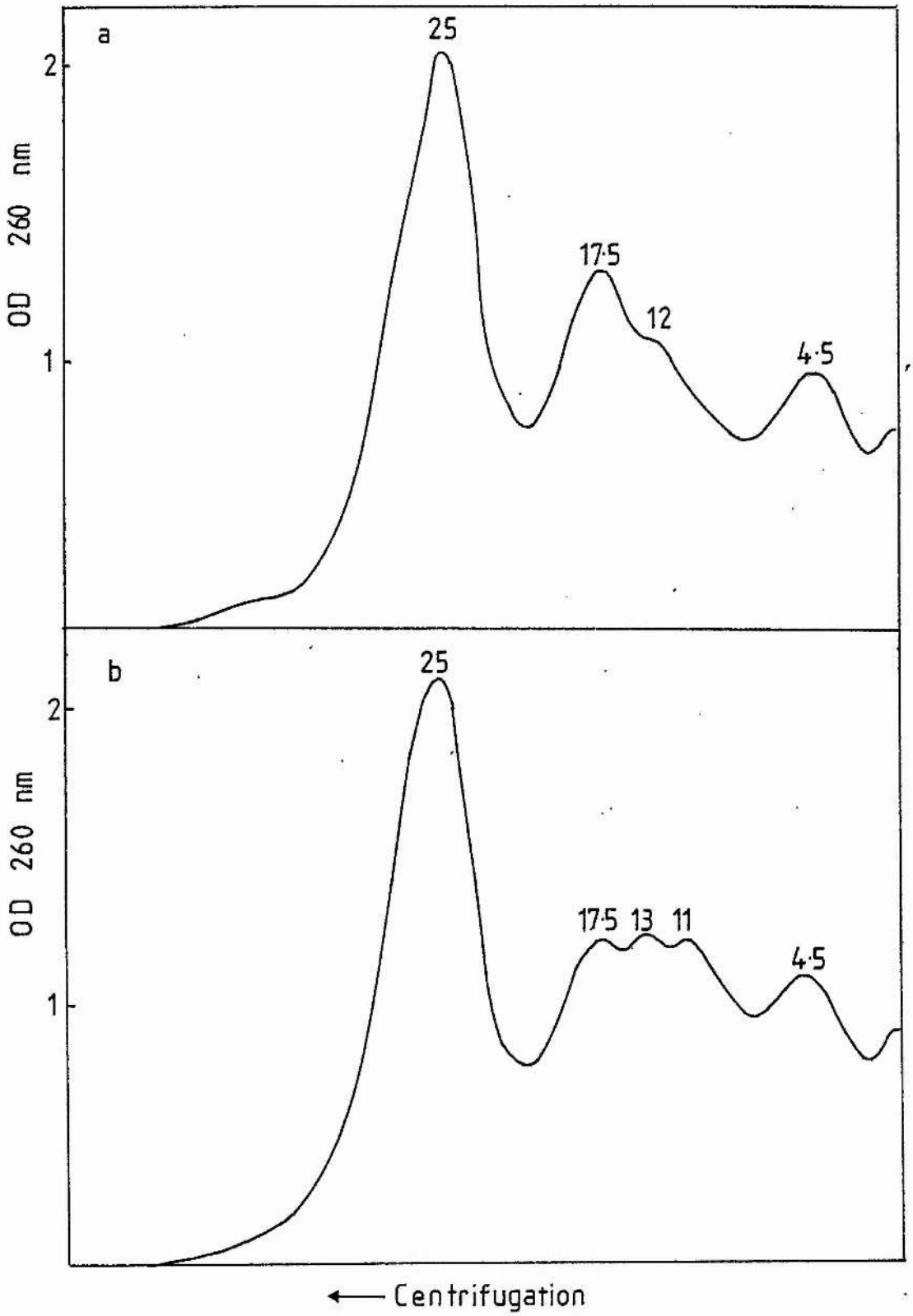

Fig. 23 a and b

Optical profiles of two samples of RNA isolated from PMS and centrifuged on a 15-30% NETS sucrose gradient.

The numbers indicate the S-value of each optical peak.

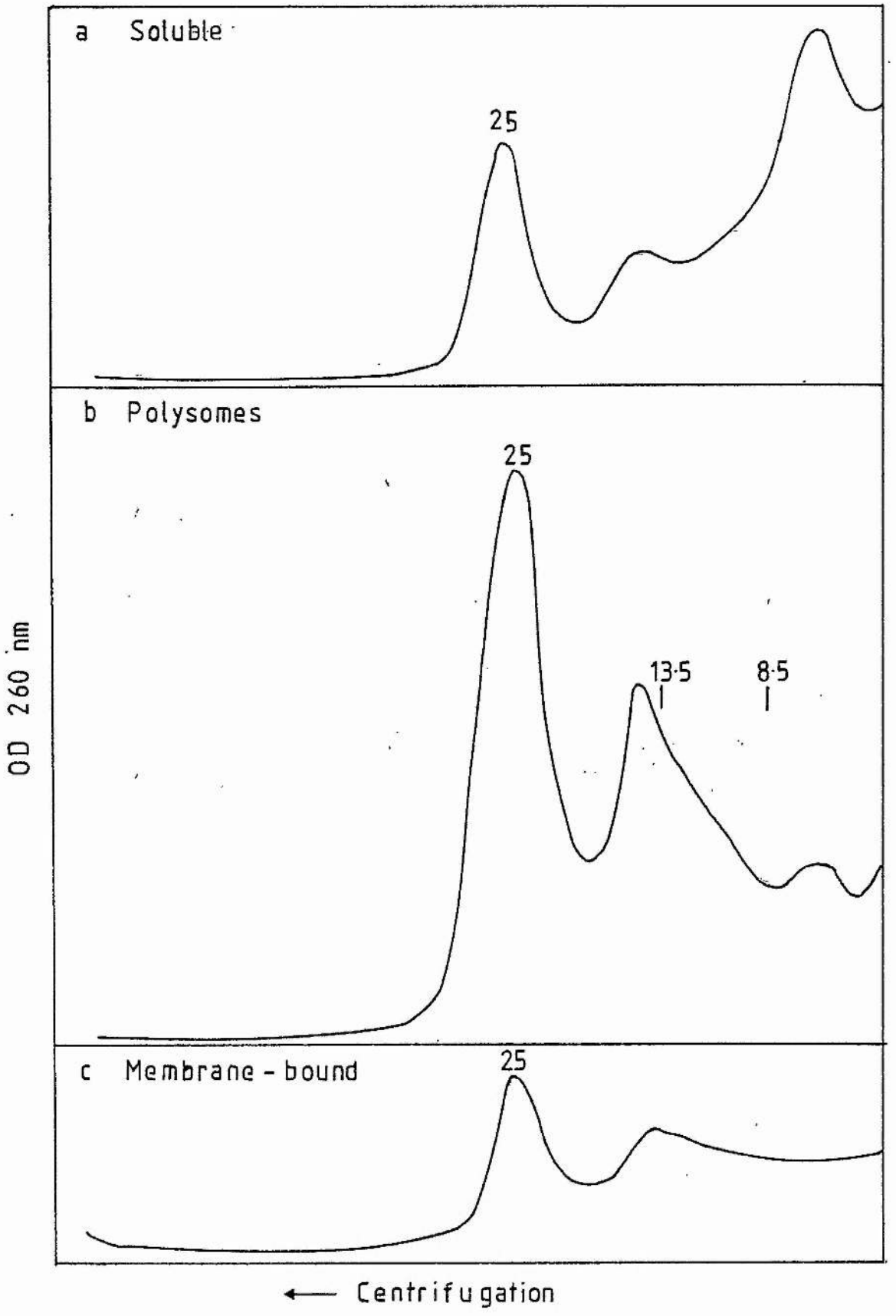

Fig. 24 a, b and c

Optical profiles of the RNA isolated from three polysome fractions.

a) Soluble fraction
b) Polysome fraction
c) Membrane-bound fraction

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RNA prepared in this way had a characteristic profile (Fig. 23a). Three peaks were always seen at 25S, 17.5S and 4.5S. These correspond respectively to the two main ribosomal RNAs and the small 5S ribosomal RNA which is not resolved from tRNA. In addition, a fourth peak or shoulder was frequently observed at 12S and this could occasionally be resolved into two separate peaks at 13 and 11S (Fig. 23b). The 12S peak is most prominent in homogenates prepared from cells which had been stored in the frozen state. In gradients in which the 12S peak was prominent there was a corresponding reduction in the 18S peak suggesting that the 12S RNA is a breakdown product of 18S RNA and that, in some way, freezing the cells stimulates this breakdown. Reisner et al. (1968) also found that the 18S RNA had a variety of breakdown products, one of which is 12.2S. Whether the 18S breakdown is due to ribonuclease activity, or to some other structural change, is not known.

RNA isolated from different parts of a polysome gradient shows different distributions of RNA species (Fig. 24 a, b and c). The top of the polysome gradient has some ribosomal RNA, possibly derived from ribosomal subunits but the main RNA class is 4 and 5S RNA. There is also a small shoulder at 11S. RNA isolated from the polysome region shows the two ribosomal RNA species and a small amount of 4 and 5S RNA which may be due to transfer RNA associated with actively translating polysomes. The most important feature is the heterogenous range of RNA between the 18 and 4 and 5S peaks. The majority of this material has a size range of 8.5-13.5S. This is probably messenger RNA and unlikely to be due to 18S RNA breakdown for

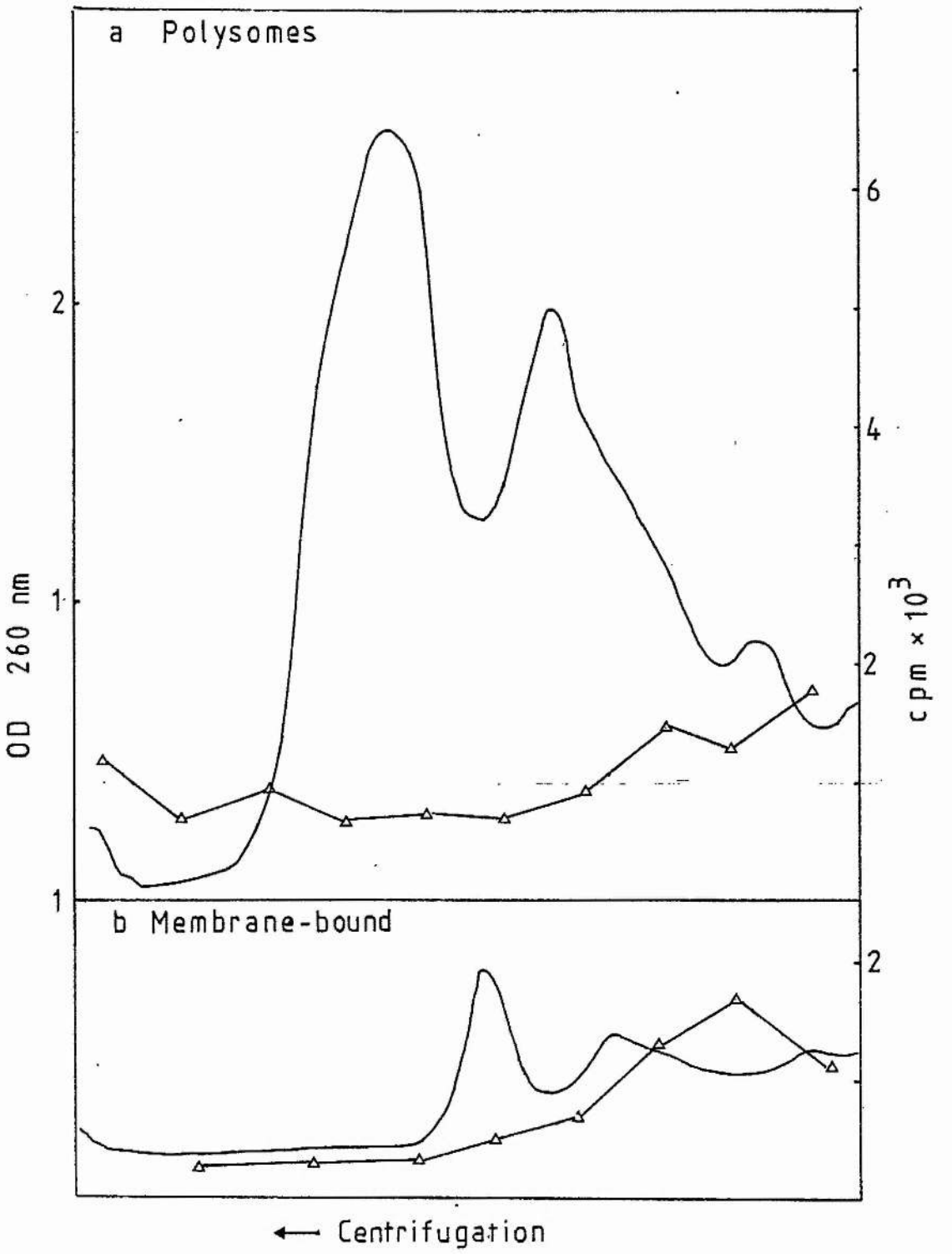

Fig. 25 a and b

Hybridisation of ^{3}H-poly(U) to gradient fractions of RNA.

a) RNA from polysomes
b) RNA from membrane-bound fraction

△ - cpm ^{3}H-poly(U) hybridised

two reasons. Firstly the ratio of 25S:18S is close to the normal 2:1 suggesting that there is little breakdown of 18S and, secondly, this material has a heterogenous size distribution rather than the characteristic 12S breakdown peak.

Although less RNA has been recovered from the membrane bound polysomes, this too shows a fraction of the RNA which has a heterogenous size distribution: here from 9-18S. The higher size range suggests that the membrane-bound fraction contains polysomes which are larger, on average, than free polysomes.

(c) Polyadenylated RNA from the postmitochondrial supernatant.

In many eucaryotes messenger RNA has been shown to have a poly(A) sequence at the 3' end (Adesnik et al., 1972). The presence of poly A^+ RNA in Paramecium can be demonstrated in two ways. Firstly, a certain proportion of RNA isolated from Paramecia, when passed through a poly(U)-sepharose or oligo(dT)-cellulose column, binds to the column. Secondly, fractions from a gradient of RNA isolated from PMS hybridise with ^{3}H-poly(U). In identical conditions of hybridisation, purified ribosomal RNA does not hybridise with ^{3}H-poly(U).

Using the second method, the distribution of polyA$^+$RNA in a gradient of RNA isolated from polysomes was examined (Fig. 25a). Apart from the bottom fraction, there is very little poly(U) hybridisation with RNA fractions larger than 18S. Most of the hybridisation is in the heterogenous RNA region, the peak of hybridisation being in the size class 9-14S. The

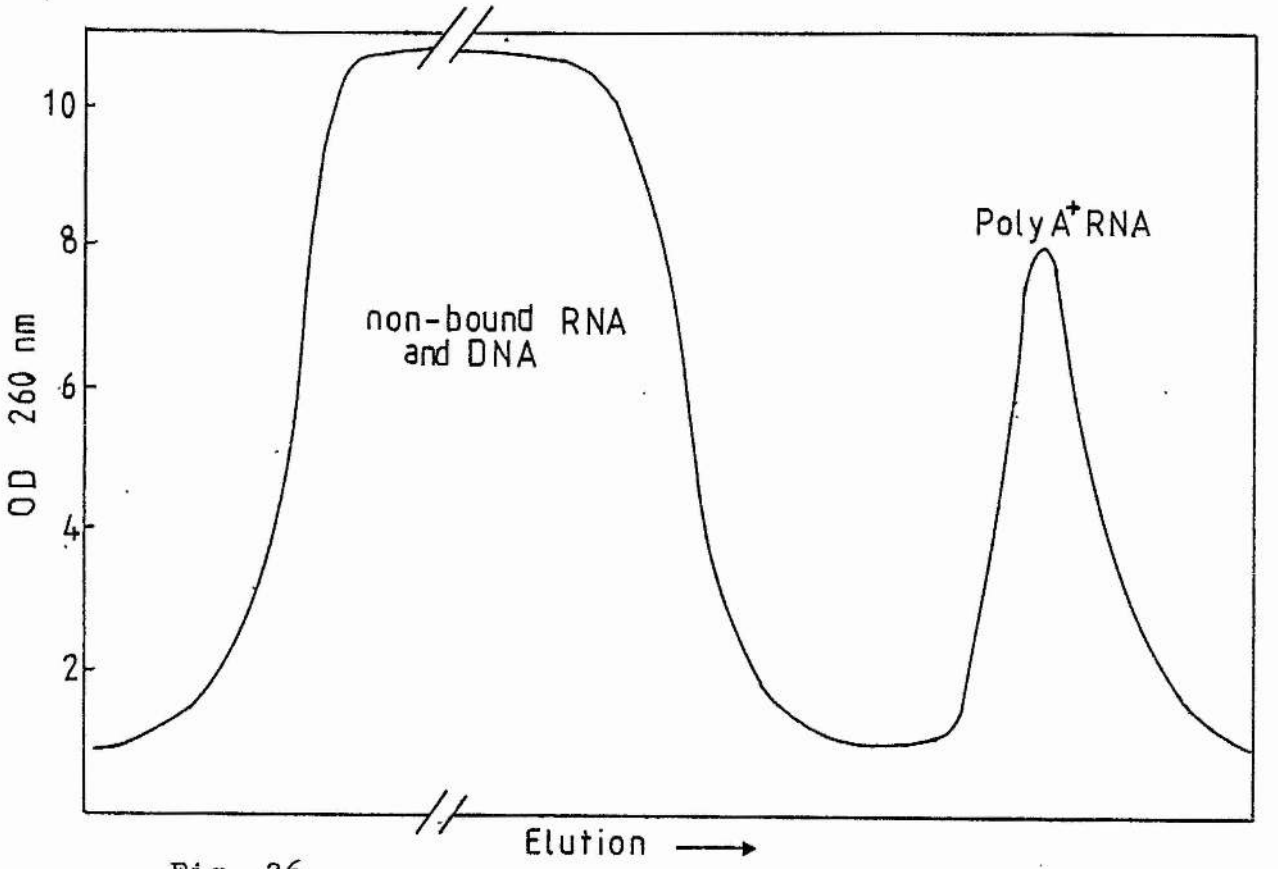

Fig. 26

Elution of polyA$^+$RNA from a column of oligo(dT)-cellulose.

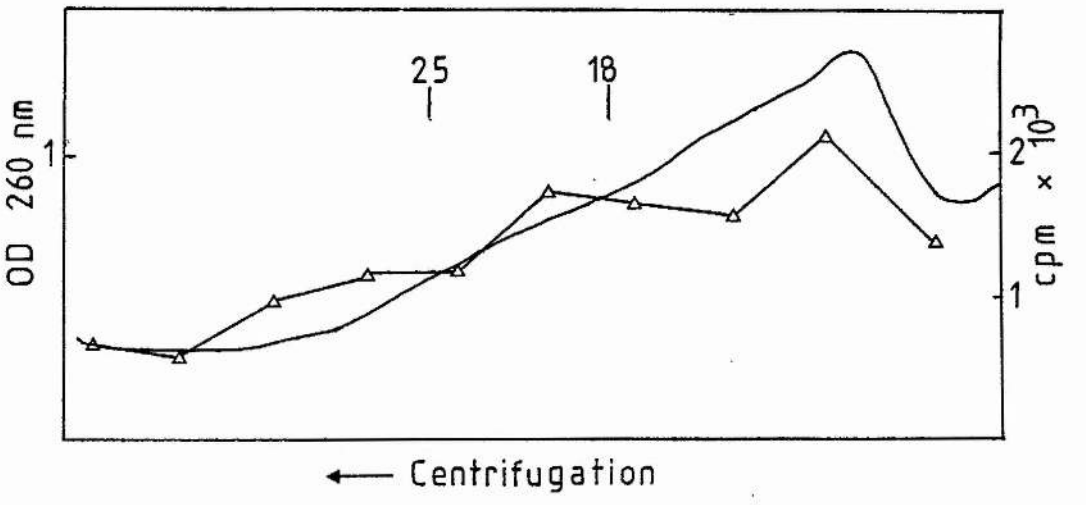

Fig. 27

Hybridisation of ^{3}H-poly(U) to gradient fractions of polyA$^+$RNA isolated from PMS

Δ - cpm ^{3}H-poly(U) hybridised
The positions of 25 and 18S RNA centrifuged on a parallel gradient are indicated.

top fraction shows some labelling, and this may be due to hybridisation of poly(U) with small amount of free poly(A).

A gradient of RNA isolated from membrane bound polysomes analysed in the same way gives similar results: most of the hybridisation is with fractions which have a size range 5-18S (Fig. 25b). There appears to be relatively more hybridisation with fractions larger than 18S than in the free polysome RNA gradient, again suggesting that the membrane bound fraction contains larger messenger RNA.

Poly-adenylated RNA was examined in more detail by isolating it on poly(U)-sepharose or oligo(dT)-cellulose columns. In conditions of high salt, polyA$^+$RNA binds to the column and, in the case of oligo(dT)-cellulose, can be eluted in conditions of low salt. In the case of poly(U)-sepharose, polyA$^+$RNA can be eluted with formamide. The elution of polyA$^+$RNA can be seen as a peak on the optical trace of material eluted from the column (Fig. 26). A small percentage of PMS RNA is bound on the first passage through a column of oligo(dT)-cellulose. On rerunning this material through the column 70% of it is rebound. Up to 1% of the total RNA could be recovered as polyA$^+$RNA after the second column binding, but the amount was variable and was sometimes as low as 0.1%.

An examination of polyA$^+$RNA isolated in this way from PMS RNA showed that, although there was a broad size distribution of material up to 30S in size, the majority was small, with a peak at 5S (Fig. 27). That this material was indeed polyA$^+$RNA, was shown by hybridising fractions from the gradient with ^{3}H-poly(U). The hybridisation roughly followed

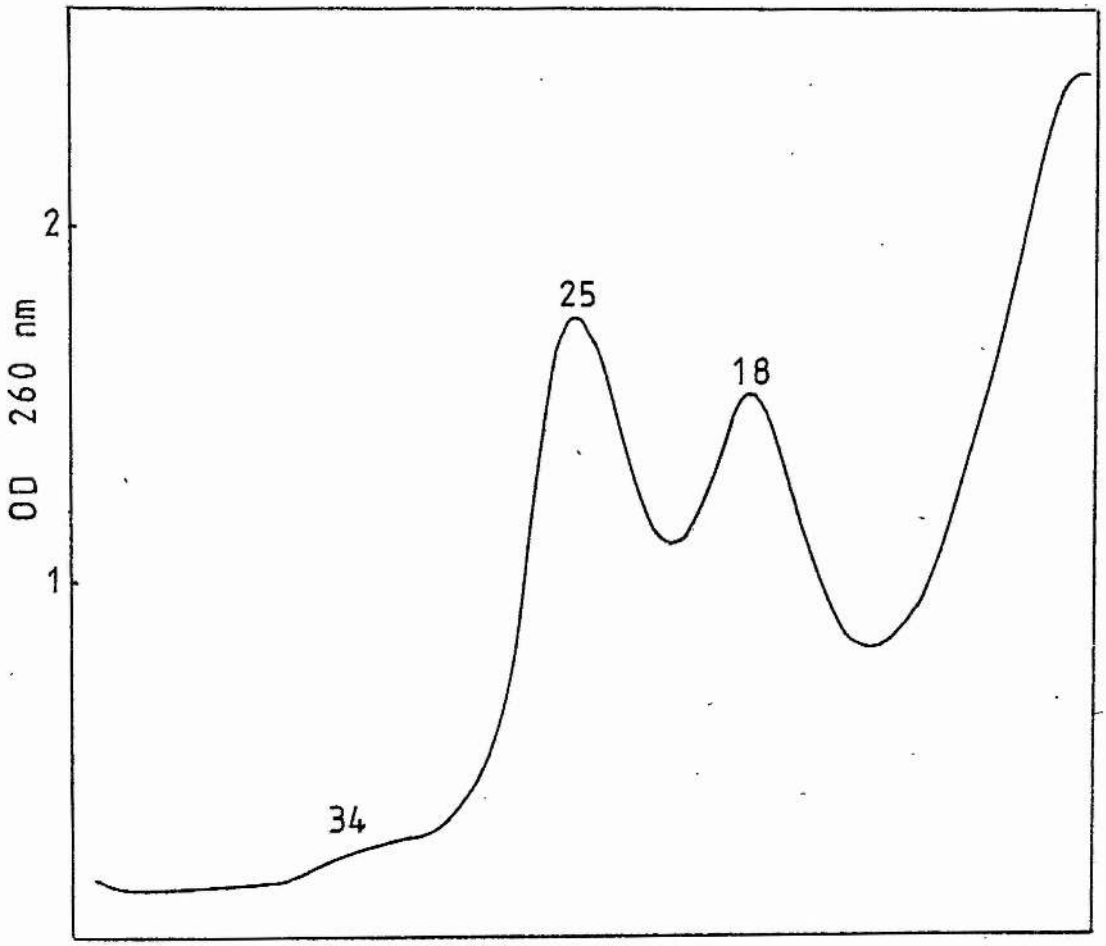

Fig. 28

Optical profile of RNA extracted by homogenisation of intact cells in Kirbys buffer. The numbers indicate approximate S-values.

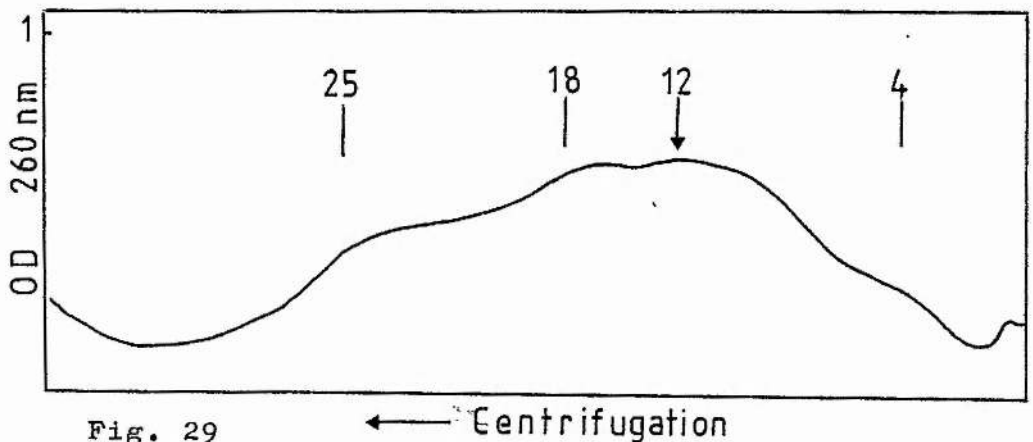

Fig. 29

Optical profile of polyA$^+$RNA isolated from Kirby-extracted RNA. The numbers indicate the positions of 25, 18 and 4S RNA on a parallel gradient. The optical peak of the polyA$^+$RNA gradient is marked with an arrow at 12S.

the optical profile. However, there appeared to be relatively more hybridisation of ^{3}H-poly(U) with fractions above 25S.

The small size of the polyA^{+}RNA, and the variability of polyA^{+}RNA yield, once more suggests low levels of ribonuclease activity. In view of this, an alternative method for isolating polyA^{+}RNA was used.

(d) RNA isolated from intact cells

The use of PMS as the source of RNA has the advantage that bacterial RNA was excluded, bacteria having been pelleted out with the mitochondria. However, for preparations of polyA^{+}RNA, the presence of bacteria could be ignored since bacterial mRNA is not poly-adenylated. RNA could therefore be prepared from intact cells. This was done by homogenising washed cells in Kirbys buffer at high pH and in the presence of sodium deoxycholate and diethylpyrocarbonate followed by phenol extraction. Kirbys buffer contains triisopropylnaphthalene sulphonate, sodium 4-amino salicylate and phenol, all ribonuclease inhibitors (Parish and Kirby, 1966) as is diethylpyrocarbonate. The presence of these inhibitors greatly improved the yield and quality of RNA isolated.

Total RNA prepared in this way had a profile similar to that of RNA prepared from the post-mitochondrial supernatant. (Fig. 28). However, the 12S peak is either absent or greatly reduced, indicating that, in the conditions of homogenisation used, 18S RNA is not so susceptible to breakdown. Another difference between the gradients of RNA isolated from PMS or from intact cells, is, in the latter, the presence of a 35S

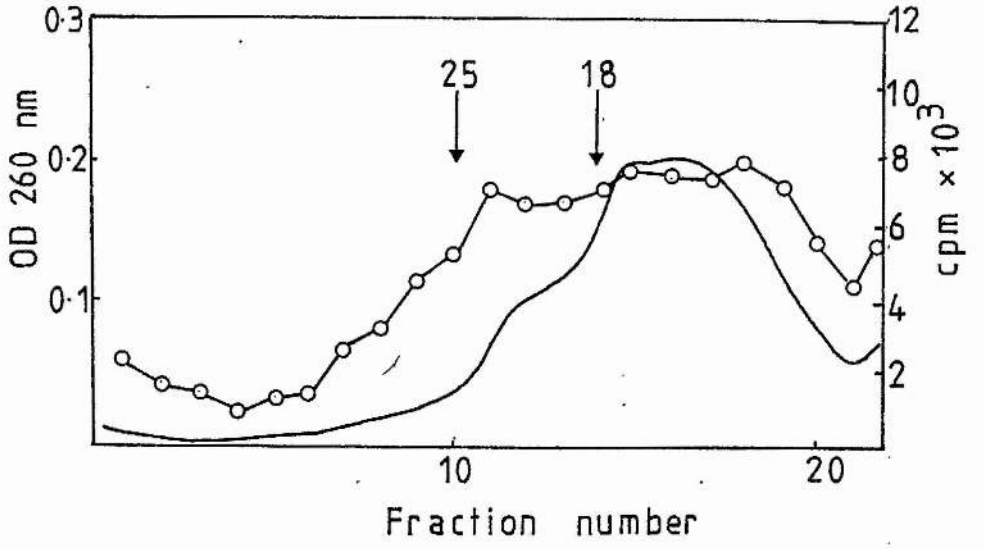

Fig. 30

Hybridisation of gradient fractions to ^{3}H-poly(U). A sample of polyA^{+}RNA isolated from Kirby-extracted cells was centrifuged on a 15-30% NETS gradient.

The positions of 25S and 18S RNAs centrifuged on a parallel gradient are indicated

o - cpm ^{3}H-polyU hybridised

Source of RNA	Sample number	% RNA as PolyA^+RNA
PMS	1	1·0%
	2	0·7%
Kirby extracted cells	1	3·1%
	2	3·4%

Table 3

The yield of polyA^+RNA from different preparations of RNA.

Sample number	% Ribonuclease resistant	calculated poly (A) length
1	10%	80 bases
2	13%	100 bases

Table 4

Poly(A) length of two samples of adenosine labelled RNA digested with ribonucleases A and T1. The calculation is based on an average polyA^+RNA length of 2000 bases and a GC content of 35% (Hruby et al., 1977).

shoulder on the 25S peak. This may correspond to ribosomal precursor RNA (Prescott et al., 1971).

(e) PolyA$^+$ RNA isolated from intact cells

PolyA$^+$RNA isolated from RNA prepared as described above differs from polyA$^+$RNA extracted from PMS in two respects. Firstly, the yield is higher (Table 3). As much as 4% can be recovered. However 2-3% was recovered from most preparations. Secondly, although this polyA$^+$RNA had a similar size range (3-40S), the majority is approximately 12-18S, with a weight average size of 2,000 nucleotides, which is much larger than polyA$^+$RNA isolated from PMS (Fig. 29).

Frequently peaks or shoulders are seen in the profile at 25S and, more commonly, at 18S indicating contamination with ribosomal RNA. This could be slightly reduced by a third passage through oligo(dT)-cellulose.

Hybridisation of polyA$^+$RNA gradient fractions with ^{3}H-poly(U) shows that, like polyA$^+$RNA isolated from PMS, polyA$^+$RNA larger than 20S in size appears to hybridise more ^{3}H-poly(U) than one might expect from the optical profile.(Fig. 30 This may indicate that larger polyA$^+$RNA molecules either have longer poly(A) tracts or contain in addition internal oligo-(A) sequences. This is a feature common to messenger precursor RNAs (Edmonds et al., 1976).

Fractions in the 18S size range show less labelling than one might expect from the optical profile and this is probably due to contamination with ribosomal 18S RNA. Fractions near the top of the gradient, i.e. smaller than 7S, show a high

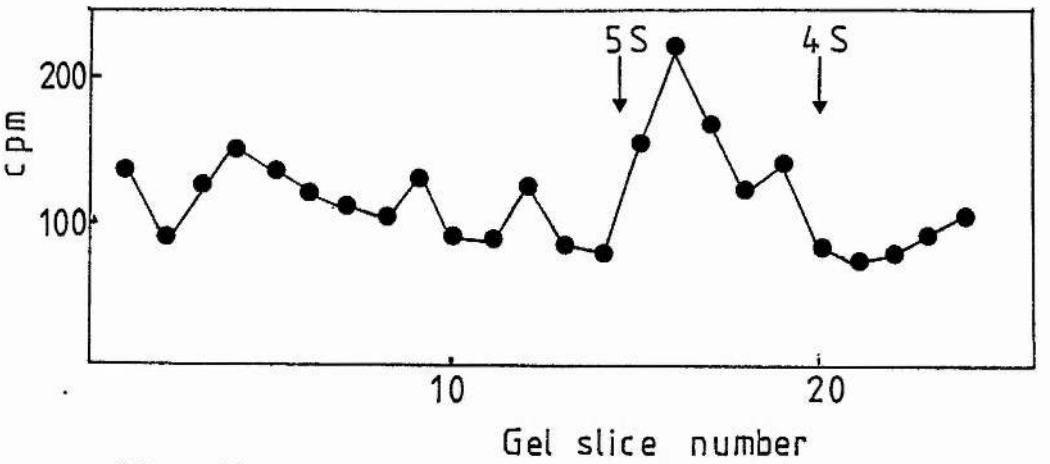

Fig. 31

Poly(A) tract gel.
^{3}H-adenosine labelled polyA^{+}RNA from Paramecium was digested with RNAse A and T1 and applied to a 15% acrylamide disc gel. 2mm slices were counted. The positions of 4S and 5S RNA on a parallel gel are indicated by arrows.

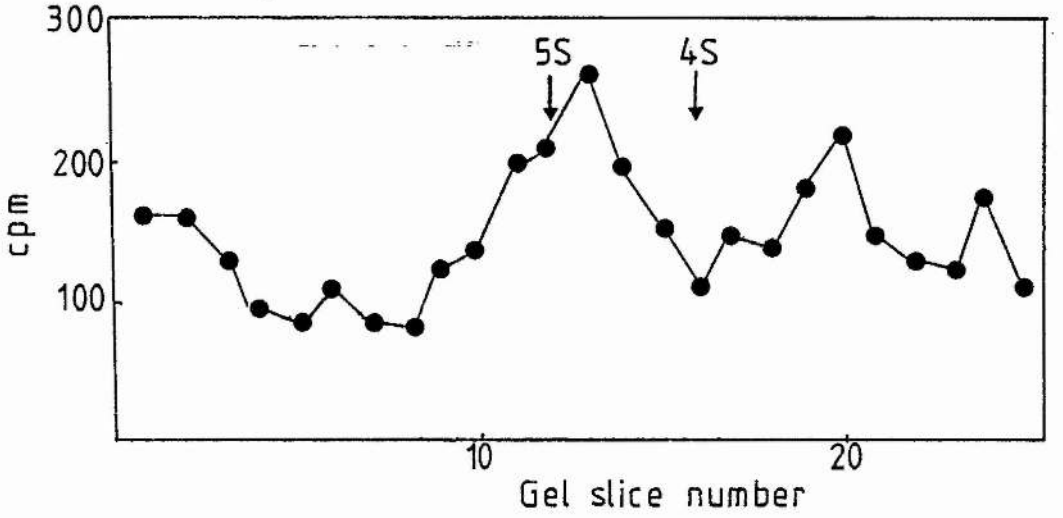

Fig. 32

A similar analysis of ^{3}H-adenosine labelled polyA^{+}RNA from Tetrahymena.

specific hybridisation. If most mRNAs have the same length of poly(A) tract, then the smaller the RNA, the greater the contribution to its length is due to the poly(A) tract, and so one might expect this effect near the top of the gradient.

(f) Length of the poly(A) tract

It has been estimated by Hruby et al. (1977) that the size of the poly(A) tract in polyA$^+$RNA from Paramecium is 250-500 bases, longer than is found in higher eucaryotes (Sheiness and Darnell, 1973). This value is derived from digestion of ^{3}H-adenosine labelled polyA$^+$RNA with ribonucleases A and T_1. However, as the authors point out, this is a rough estimate. An accurate estimate depends on having ^{3}H-adenosine labelled polyA$^+$RNA of a known size distribution free from contaminating polyA$^-$RNA. The method used to calculate the poly(A) tract length also assumes that all poly(A) stretches are the same length and that all ribonuclease resistant material is indeed poly(A) tract and not internal oligo-(A) sequences.

A similar digestion of two preparations of ^{3}H-adenosine-labelled polyA$^+$RNA with the ribonucleases A and T_1 gives values of 10% and 13% resistance respectively. The A content of polyA$^+$RNA is 35% (Hruby et al., 1977). Assuming that the average length of polyA$^+$RNA is 2000 nucleotides, then the poly(A) tract length is 80-100 bases (Table 4).

Again this is a rough estimate. A more accurate method to measure the poly(A) tract length would be acrylamide gel electrophoresis of ribonuclease resistant material. Relatively little radioactivity has been incorporated in this material, but there appears to be a peak of radioactivity slightly smaller than the 5S marker (Fig. 31). ^{3}H-adenosine labelled polyA$^+$RNA from Tetrahymena examined in the same way shows two

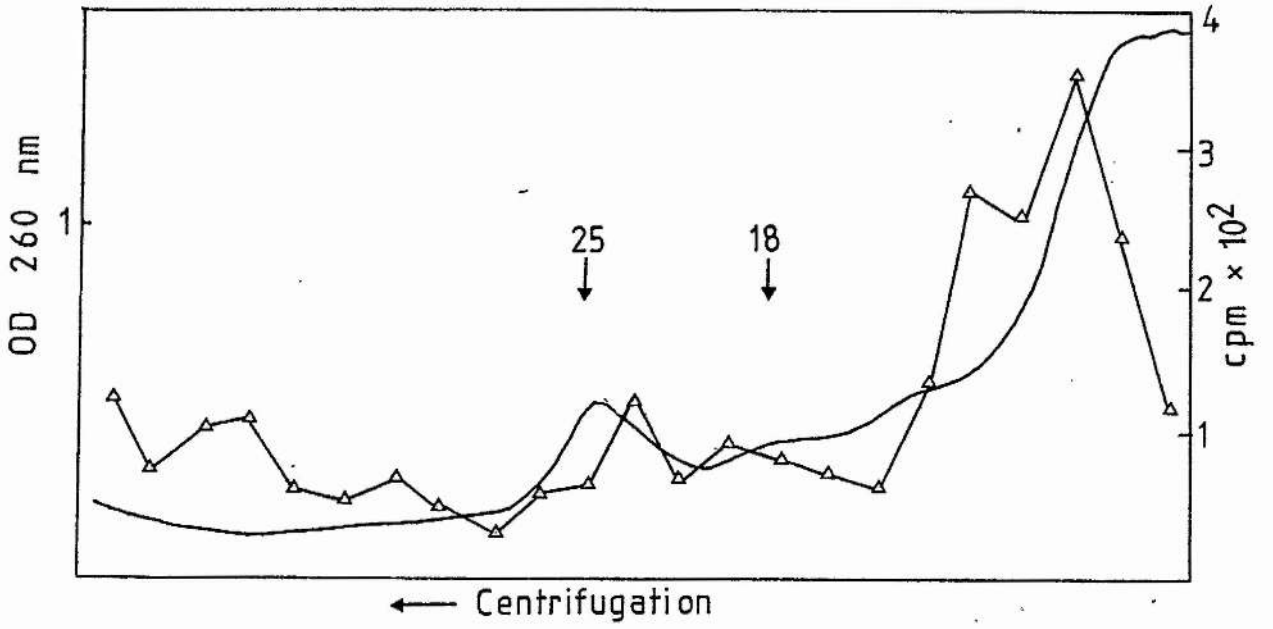

Fig. 33

Optical profile of RNA isolated from macronuclei and hybridisation of ^{3}H-poly(U) to gradient fractions.

△ - cpm ^{3}H-poly(U) hybridised

peaks: one at 5S and one slightly smaller than 4S (Fig. 32). The former is probably the poly(A) tract of cellular mRNA, and the latter may be the shorter polyA tract of mitochondrial mRNA, which in Hela cells has a length of 50-80 bases (Perlman et al., 1973).

If the two species of ciliate are comparable with respect to poly(A) tract length, it would appear that the ciliates have poly(A) tracts 100 ± 20 bases in length, slightly shorter than is found in most higher eucaryotes (e.g. Darnell et al., 1971).

(g) RNA isolated from macronuclei

The optical profile shows that the major RNA species in macronuclei is 25S (Fig. 33). This is in agreement with the early results of Cummings (1972). In addition the 18S species is in evidence but no 35S ribosomal precursor can be seen. There is a considerable amount of material at the top of the gradient. Whether this reflects the in vivo condition or is due to RNA breakdown during nuclear isolation is not known. The latter explaination is likely to be the case since, while transcription units can be observed in spread preparations of intact cells, none are seen in similar preparations of isolated macronuclei (see Chapter III iii).

Fractions from the gradient have been hybridised with ^{3}H-poly(U) to detect poly(A) containing RNA. Much of the shorter material in the gradient hybridises ^{3}H-poly(U), a peak at 9S being seen. In addition RNA in the size range 20-22S hybridises ^{3}H-poly(U). There is also a significant amount of hybridisation in the 40S region, more than one finds in cytoplasmic RNA, and may represent a type of hnRNA.

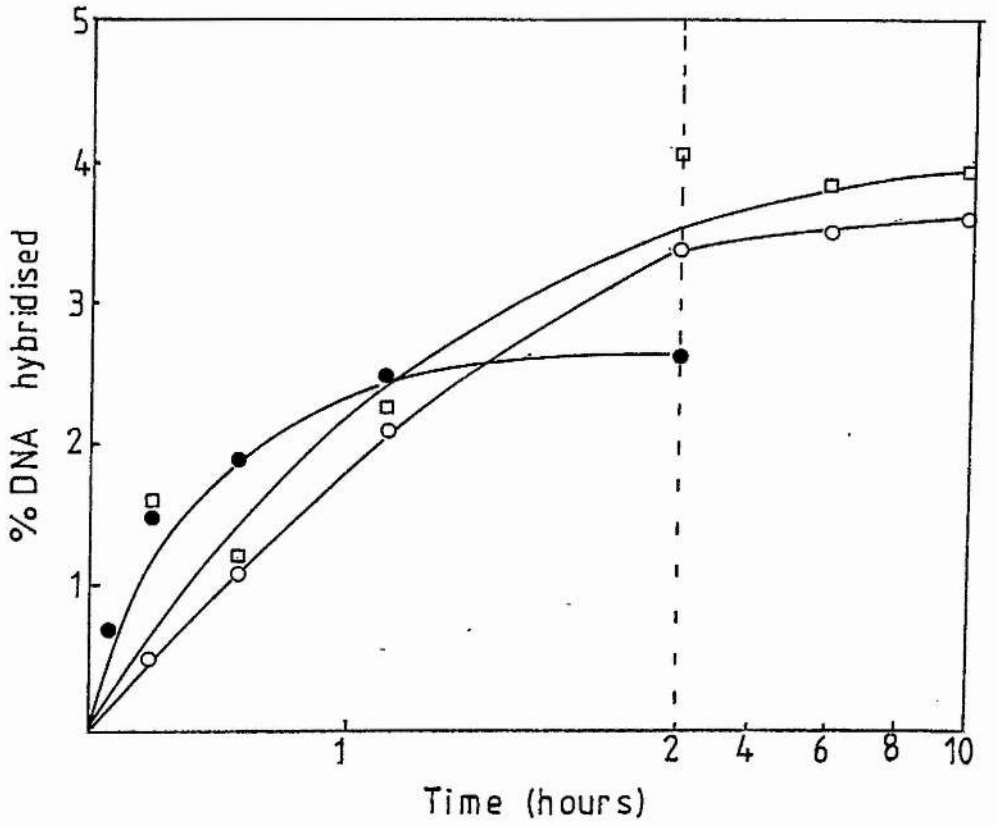

Fig. 34

Time course of hybridisation of ^{125}I-DNA with polyA$^+$RNA.

● - G polyA$^+$RNA

○ - D polyA$^+$RNA

□ - A mixture of equal quantities of G and D polyA$^+$RNA

The dotted line indicates a change in time-scale. The data are corrected for DNA self-reassociation as determined from controls containing DNA alone.

(ii) Sequence Complexity of PolyA$^+$RNA

In any one tissue of higher eucaryotes, only a small percentage of the DNA is transcribed into polyA$^+$RNA (e.g. Turner and Laird, 1973., Ryffel and McCarthy, 1975., Bantle and Hahn, 1976.). Since the macronuclear genome of Paramecium is relatively simple, one might expect that proportionately more of the DNA might be transcribed. Dictyostelium, which has a similar genetic complexity, transcribes 56% of the genome (Firtel, 1972).

(a) Percentage of DNA transcribed

A sample of macronuclear DNA was iodinated, denatured and hybridised at 55°C with an excess of polyA$^+$RNA isolated from cells grown at 25°C (G cells), cells grown at 32°C (D cells) or with a mixture of polyA$^+$RNA isolated from both cell types. Samples were hybridised for various lengths of time and were assayed by S_1 - nuclease digestion. The iodinated denatured DNA is not entirely susceptible to S_1 nuclease: 3% remains undigested. This value has therefore been subtracted from all sample values.

A vast excess of polyA$^+$RNA from G cells hybridises to ^{125}I-DNA and gives a saturation value of 2.5% after 2 hours, by which time a Rot of 80 has been reached (Fig. 34). By the same Rot value, polyA$^+$RNA from D cells has hybridised with ^{125}I-DNA and gives a saturation value of 3.4%. The rate of hybridisation is slower and clearly a plateau value has not been reached by this Rot value. Two further samples, however, show that the saturation value is only slightly higher, at

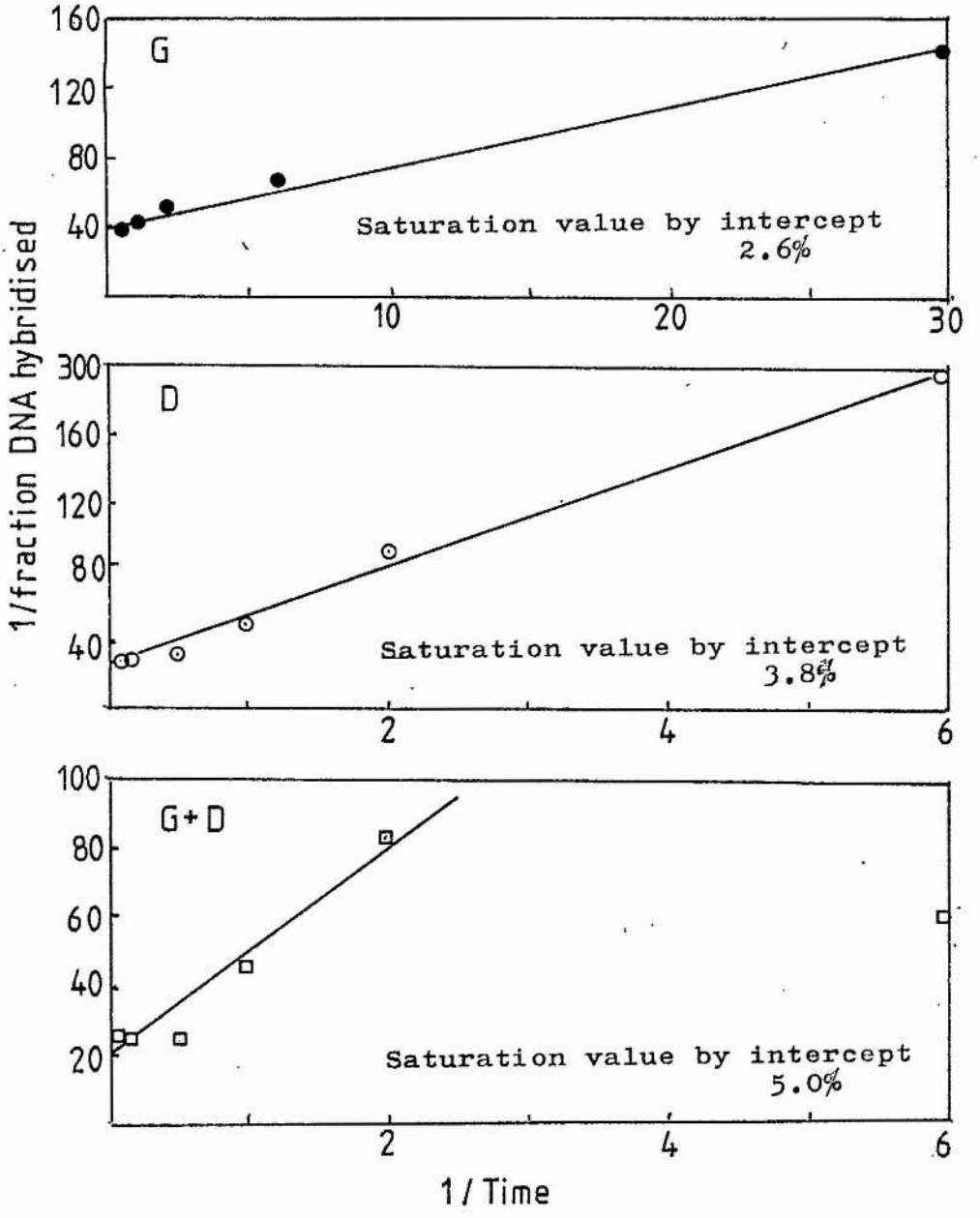

Fig35a, b and c

Inverse plot of the data of Fig. 34.

● - G

○ - D

□ - G and D

approximately 3.5%. With a mixture of equal amounts of the two polyA$^+$RNAs, although the initial points do not fall on the expected curve, the two final points both give the same value of 3.8%.

The saturation value can be more accurately determined from the intercept value of an inverse plot of the data (Wetmur and Davidson, 1968., Bishop, 1969) (Fig. 35). The values, by this method, are the same for polyA$^+$RNA from G cells, but slightly higher for D polyA$^+$RNA and the mixture of RNAs, indicating that these RNA samples contain RNA sequences at low frequencies which hybridise the ^{125}I-DNA at a slow rate, accounting for the slight increase in saturation values at longer time intervals. The values of 2.6%, 3.8% and 5% for G polyA$^+$RNA, D polyA$^+$RNA and G + D polyA$^+$RNA respectively mean that 5.2% of the macronuclear genome is transcribed into polyA$^+$RNA at 25°C (assuming assymetric transcription) (Table 5). At the higher temperature of 32°C, 7.6% of the macronuclear genome is transcribed. That some of the polyA$^+$RNA sequences transcribed at the lower temperature is the same, is shown by the saturation value for a mixture of RNAs which is 10.0%. Were the polyA$^+$RNA sequences in G and D cells totally different, the saturation value for the mixture would be 12.8%.

From the values, one can calculate that 2.8% of the macronuclear genome is transcribed into polyA$^+$RNA sequences which are present in both G and D cells. A further 2.4% is transcribed in G cells only, and a further 4.8% in D cells only.

	% by saturation	% by intercept	% of genome
G	2·6 %	2·6 %	5·2 %
D	3·5 %	3·8 %	7·6 %
G+D	3·8 %	5·0 %	10·0 %
common sequences	2·3 %	1·4 %	2·8 %
G only sequences	0·3 %	1·2 %	2·4 %
D only sequences	1·2 %	2·4 %	4·8 %

Table 5

Values from Figs. 34 and 35

In column 1, the values are derived from Fig. 34

In column 2, the values are derived from Fig. 35

In column 3, the values are derived from column 2, assuming assymmetric transcription of the DNA.

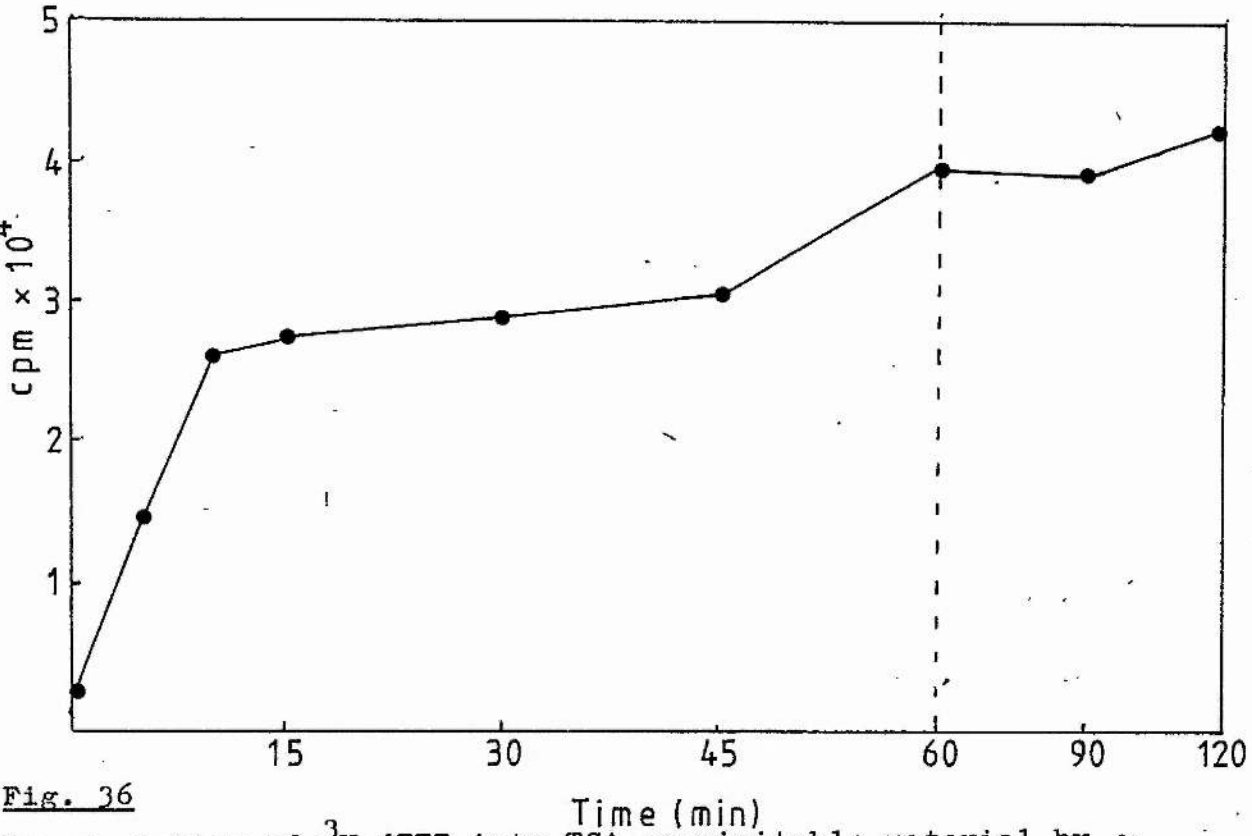

Fig. 36

Incorporation of ^{3}H-dTTP into TCA precipitable material by a sample of reverse transcriptase with poly(A) as the template.

● - Reverse transcriptase from N.I.H.

The dotted line indicates a change in time-scale.

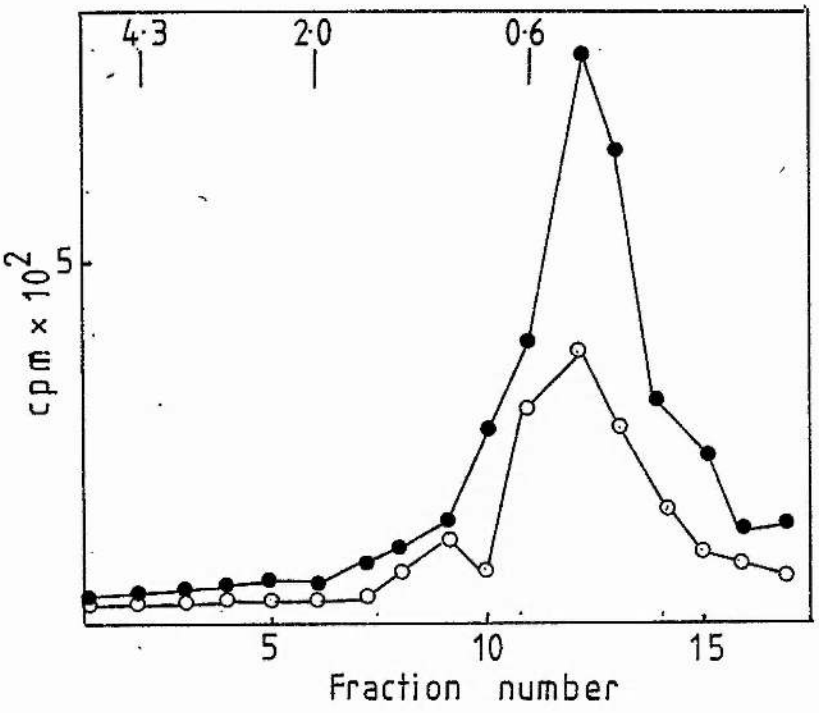

Fig. 37

Alkaline sucrose gradient analysis of two samples of cDNA. The position of marker DNAs (Hind III digest of λ DNA) are indicated. Values are in kilobases.

● - G cDNA

○ - D cDNA

Since the macronuclear genome complexity is 1.6×10^8 base pairs, the number of different polyA$^+$RNA sequences in each situation can be calculated, assuming that the weight average of polyA$^+$RNA molecules is 2×10^3 nucleotides. From this calculation, it can be said that G cells transcribe approximately 4×10^3 different polyA$^+$RNA molecules, of which half are also transcribed by D cells. D cells transcribe 6×10^3 different polyA$^+$RNA molecules of which one third are also transcribed in G cells.

These values can be considered to be only approximate for two reasons. Firstly, it is difficult to measure accurately the saturation value when only a small percentage of the iodinated DNA probe is hybridised. Furthermore, sequences present at low concentrations, which consequently hybridise to the ^{125}I-DNA at a slow rate may not be detected within the time-course of a saturation experiment, even if they constitute a large percentage of the complexity. the first problem can be overcome by the use of cDNA. A reverse transcribed cDNA copy of the polyA$^+$RNA can be hybridised with an excess of the polyA$^+$RNA template. Hence, theoretically, 100% of the cDNA probe can be hybridised, although usually only 75-90% is actuallyhybridised(Bishop et al.,1974).

(b) Preparation of cDNA

Reverse transcriptase supplied by Dr.J.Beard, N.I.H. Bethesda, was used to prepare cDNA. Normally both dCTP and dTTP were used as labelled nucleotides,and the incubation carried out for 1hr.(Fig.36). The cDNA was routinely sized on an alkaline sucrose gradient and compared with a similar gradient of Hind 111 digested λ DNA(Boehringer). cDNA prepared from G and D polyA$^+$RNA and from rabbit reticulocyte polyA$^+$RNA was in the size range 400-500 bases (Fig.37).

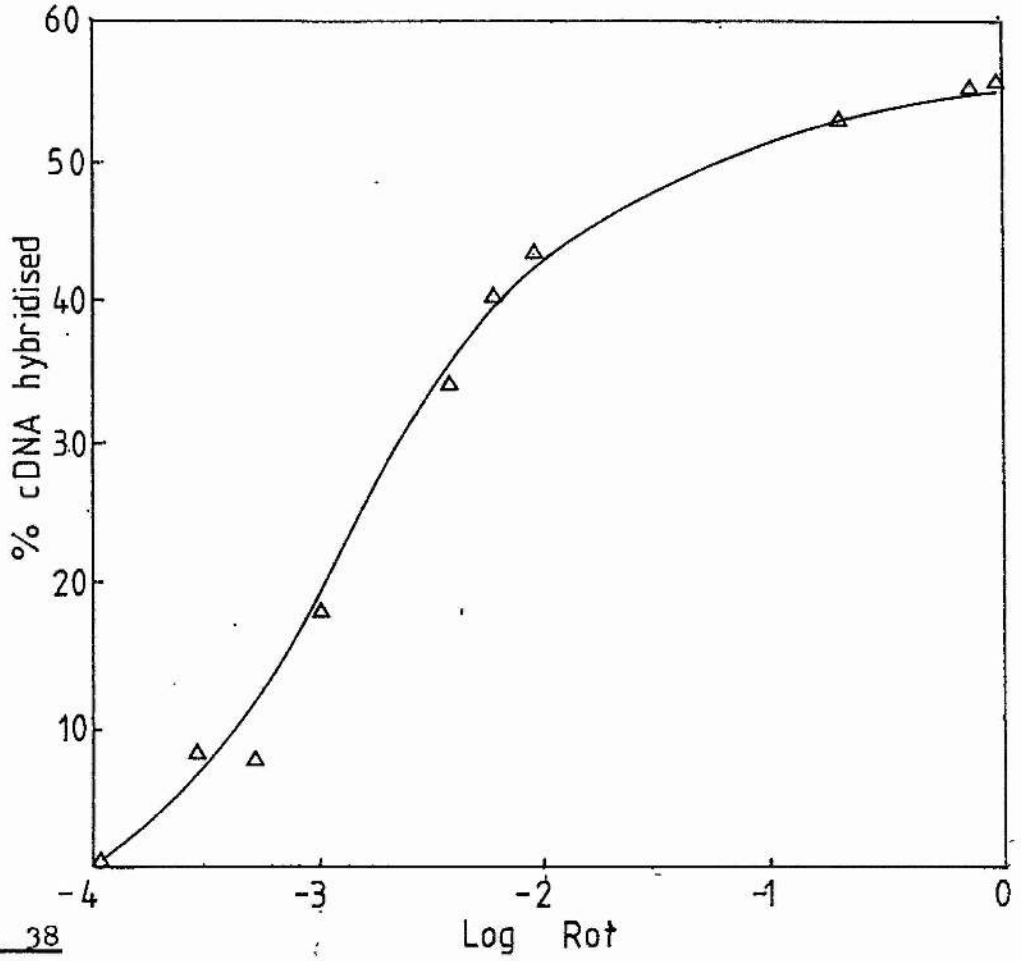

Fig. 38

Hybridisation of rabbit reticulocyte polyA$^+$RNA with its cDNA copy.

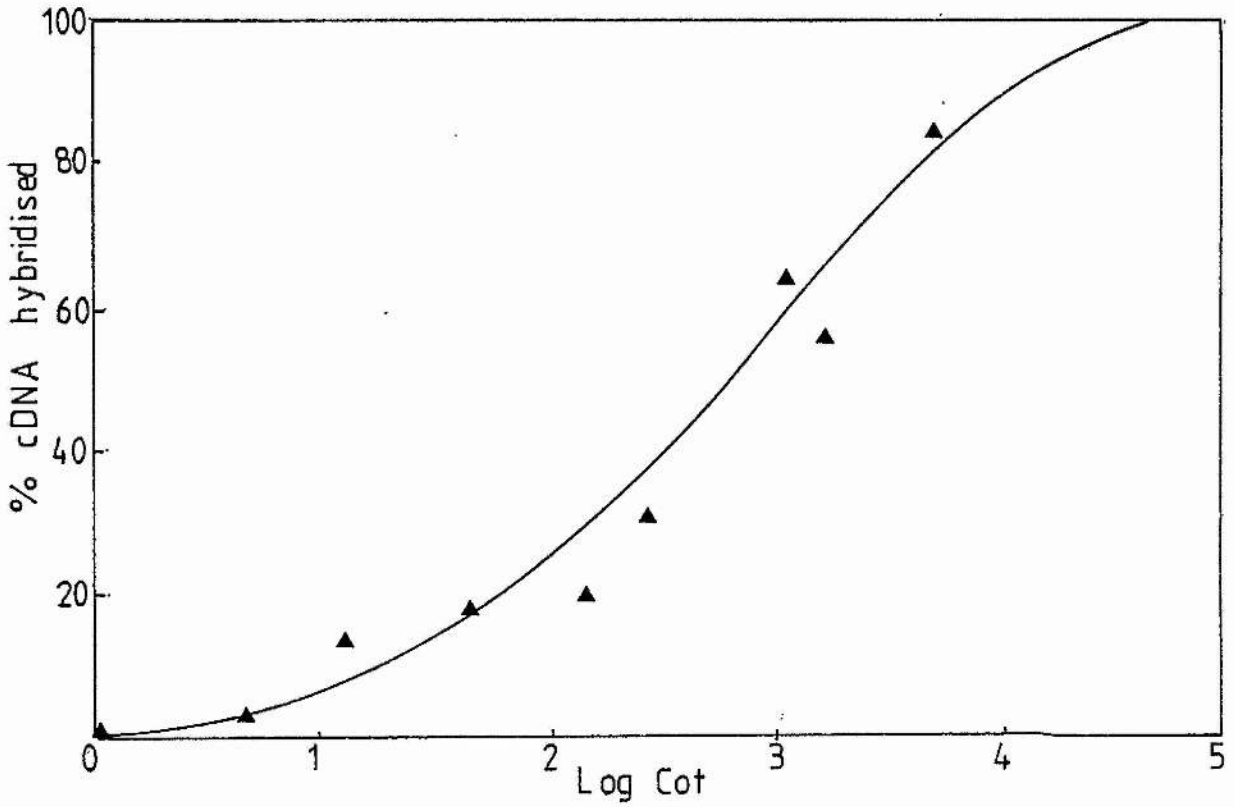

Fig. 39

Hybridisation of macronuclear DNA with cDNA prepared to G polyA$^+$RNA

(c) Complexity of rabbit reticulocyte polyA$^+$RNA

In order to provide a standard for comparison with Paramecium polyA$^+$RNA complexity, cDNA was prepared from polyA$^+$RNA isolated from the blood of a rabbit stimulated to produce a high level (40%) of reticulocytes. The cDNA was hybridised with excess of the polyA$^+$RNA (Fig. 38). The curve is not an ideal first-order kinetic curve and less than 60% of the cDNA has been hybridised by a Rot value of 1. This effect is probably due to the presence of mRNA sequences other than globin polyA$^+$ RNA. Therefore non-globin mRNA might account for as much as 30% of the RNA. The Rot$\frac{1}{2}$ value can be corrected for this, giving a Rot$\frac{1}{2}$ value for globin polyA$^+$RNA-cDNA hybridisation of 1.6×10^{-3}, which is similar to values obtained by other workers (Gummerson and Williamson, 1974), although different conditions of incubation and assay have been used here.

Globin polyA$^+$RNA consists of molecules coding for α - globin and β - globin. The molecular weight of globin mRNA is about 2×10^5 daltons (Carter and Kraut, 1974). A Rot$\frac{1}{2}$ value of 1.6×10^{-3} is therefore taken to represent a RNA sequence complexity of 4×10^5 daltons or 1200 nucleotides.

(d) Hybridisation of cDNA to polyA$^+$RNA from G cells to DNA

Macronuclear DNA appears to consist largely of uniquely represented sequences (see Chapter II). However, it is possible that a disproportionate amount of polyA$^+$RNA could be transcribed from a small amount of repetitive DNA which may

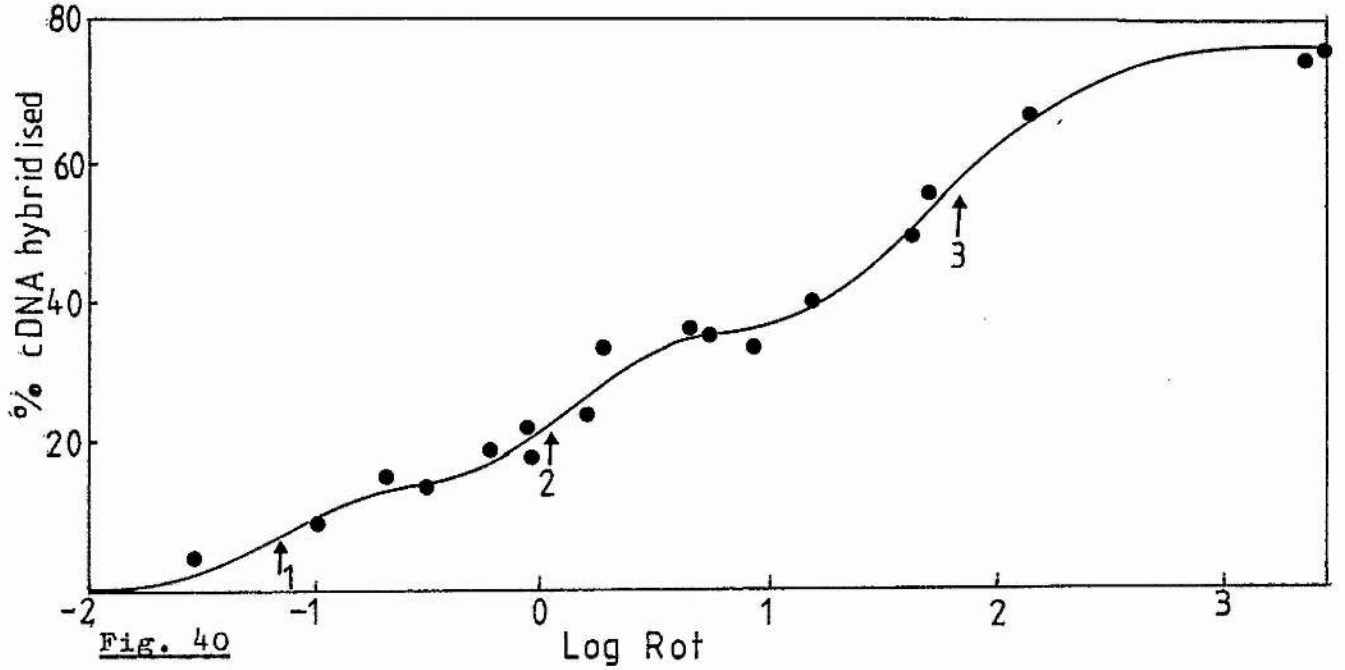

Fig. 40

Hybridisation of G polyA$^+$RNA with its cDNA copy. The arrows indicate the Rot$\frac{1}{2}$ of the 3 transitions numbered 1, 2 and 3.

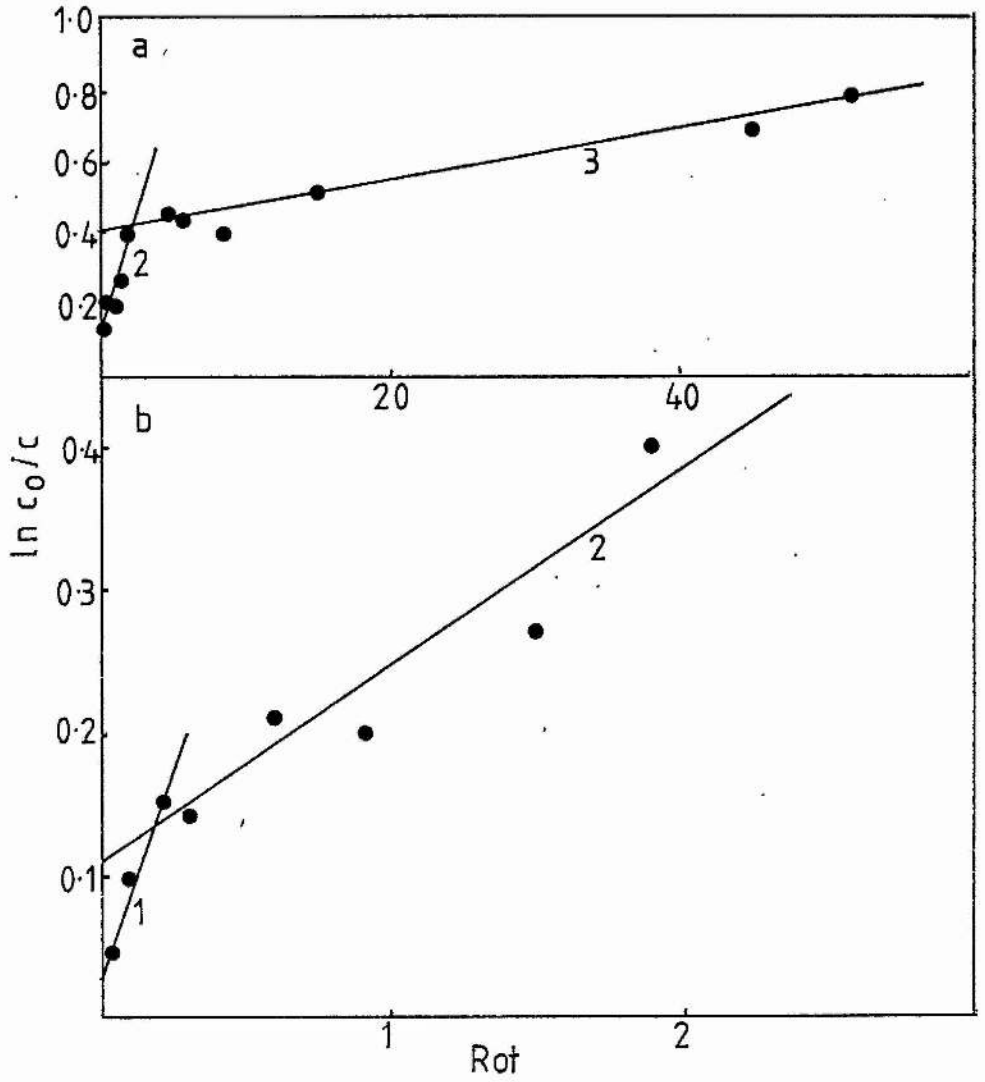

Fig. 41 a and b Linear plot of the data in Fig.40 The numbers correspond to the transitions denoted in Fig.40

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be present in macronuclear DNA. In order to ascertain whether or not this is the case, cDNA prepared to polyA$^+$RNA from G cells was hybridised to an excess of unlabelled macronuclear DNA. As seen from Fig. 39, the kinetics of cDNA-DNA are similar to those of ^{125}I-DNA - DNA hybridisation, indicating that most, if not all, of the polyA$^+$RNA is transcribed from unique DNA.

(e) Complexity of Paramecium polyA$^+$RNA from G cells

PolyA$^+$RNA isolated from cells grown at 25°C (G cells) was hybridised in excess with its cDNA copy. Fig. 40 shows that the hybridisation reaction is complex, the cDNA hybridising to polyA$^+$RNA over a wide range of Rot values, indicating that polyA$^+$RNA species are present at different intracellular concentrations. This pattern of hybridisation is found in RNA extracted from many types of eucaryotic cells or tissues (e.g. Bishop et al., 1974., Ryffel and McCarthy, 1975., Hereford and Rosbash, 1977).

Two transitions are evident in Fig. 40 but, for the purpose of analysis, the first transition has been regarded as the sum of two separate first-order curves. The data has been analysed using a linear plot (Wetmur and Davidson, 1968., Bishop et al., 1974., Hereford and Rosbach, 1977). In Fig. 41 a and b the natural logarithm of the inverse of the proportion of cDNA remaining single-stranded (ln Co/C) has been plotted against Rot. The percentage of cDNA involved in each transition has been determined from the intercept values and the Rot$\frac{1}{2}$ has been derived from the slope of the line and the intercept value. Further analysis of the data is shown in

Table 6 Numerical analysis of the data from Figs. 40-43

	Trans. No.[a]	Intercept[b]	Slope[b]	% cDNA[c]	Rot$\frac{1}{2}$[d]	corrected Rot$\frac{1}{2}$[e]	sequence complexity[f]	No. of RNA sequences[g]	No. of molecules per cell[h]	% of genome complexity[i]
	1	0.025	0.58	13%	0.069	0.0056	4.2×10^3	2	3.7×10^6	0.0026%
G	2	0.105	0.135	31%	1.1	0.21	1.6×10^5	80	2.3×10^5	0.1%
	3	0.40	0.007	56%	71	25	1.87×10^7	9,000	3.5×10^3	11.7%
	1	0.01	1.14	13%	0.040	0.0036	2.7×10^3	1	5.7×10^6	0.0017%
D	2	0.10	0.105	40%	1.9	0.48	3.6×10^5	180	1.3×10^5	0.23%
	3	0.51	0.0028	47%	157	46	3.5×10^7	17,500	1.6×10^3	22%

Notes
a) The transition number refers to the components numbered in Figs. 40-43
b) The slope and intercept are derived from Figs. 41 and 43.
c) The percentage cDNA is calculated from the intercept values
d) The Rot$\frac{1}{2}$ is calculated from the slope and intercept, assuming a plateau value of 75%
e) The Rot$\frac{1}{2}$ is corrected for % cDNA, a GC content of 35% and a percentage poly(A) content of 1%
f) The sequence complexity is calculated by comparison of the Rot$\frac{1}{2}$ with the Rot$\frac{1}{2}$ of globin polyA$^+$RNA - cDNA hybridisation (Fig. 38)
g) The number of RNA sequences is based on the sequence complexity value and assuming the average length of a polyA$^+$RNA molecule to be 2000 nucleotides
h) The number of molecules per cell is based on a polyA$^+$RNA content of 67.5pg/cell
i) The percentage of the genome complexity is derived by a comparison of the RNA sequence complexity with a genome sequence complexity of 1.6×10^8 nucleotides.

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Table 6.

For G cells, approximately 55% of the cDNA hybridises with a Rot$\frac{1}{2}$ of 70. This constitutes the least abundant class of RNA sequences and it can be calculated that it consists of 9000 different sequences of polyA$^+$RNA with average size 2000 nucleotides. This constitutes 11.7% of the genome complexity, higher than the value derived by saturation.

The two earlier transitions, constituting RNA species present at high concentrations in the cell, account for 13 and 31% of the cDNA respectively. After correcting for a number of factors (see Table 6), the sequence complexities of these components are 4.2×10^3 nucleotides and 1.6×10^5 nucleotides, and would correspond to 2 mRNA species and 80 mRNA species respectively.

It is estimated that a cell contains 2200-3210pg of RNA (Skoczylas and Soldo, 1975). Of this 2-3% can be recovered as polyA$^+$RNA i.e. 67.5 pg or 1.2×10^{11} nucleotides of polyA$^+$RNA per cell. From this it can be calculated that each cell contains 3.7×10^6 molecules of the most frequent class of RNA sequences, 2.3×10^5 molecules of the intermediate frequency class and 3.5×10^3 molecules of the least frequent class. This latter value is much higher than the 1-10 molecules for the low frequency class found in other cells and may reflect the polyploid nature of the macronucleus.

It must be stressed, however, that these values are very approximate. Firstly, it is difficult to derive the Rot$\frac{1}{2}$ and percentage of each component when the points

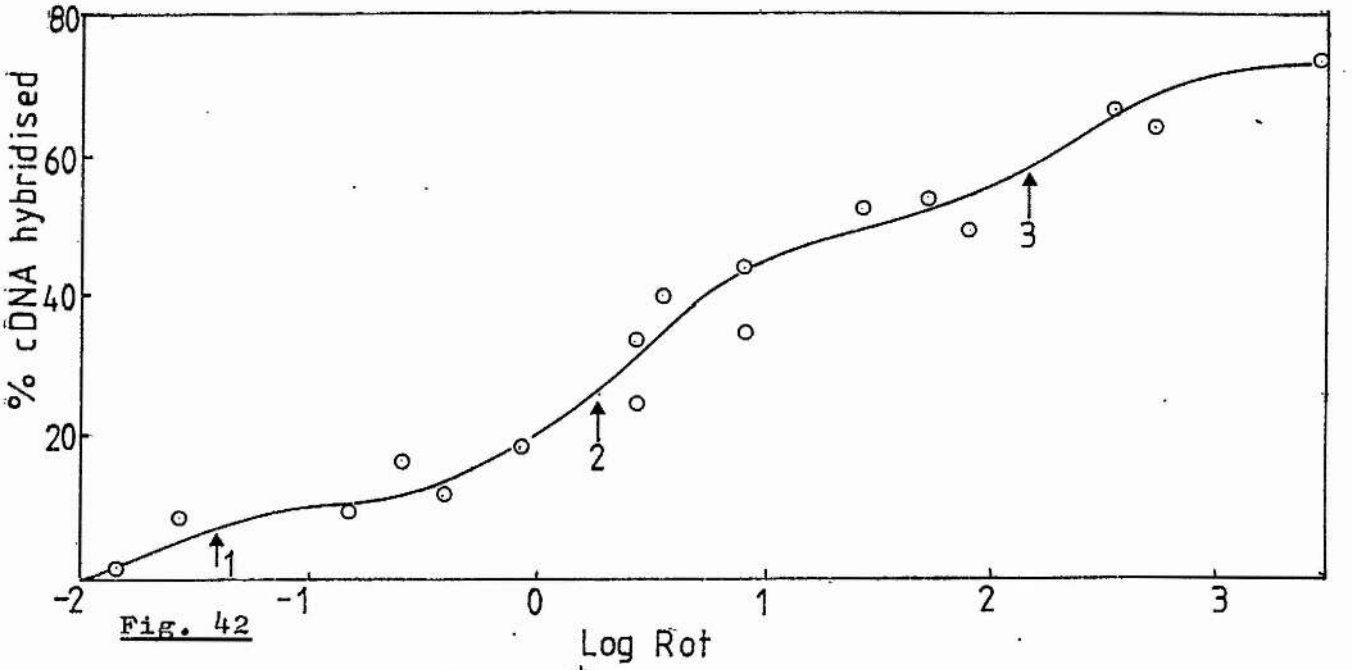

Fig. 42

Hybridisation of D polyA$^+$RNA with its cDNA copy. The arrows indicate the Rot$\frac{1}{2}$ of the 3 components numbered 1, 2 and 3.

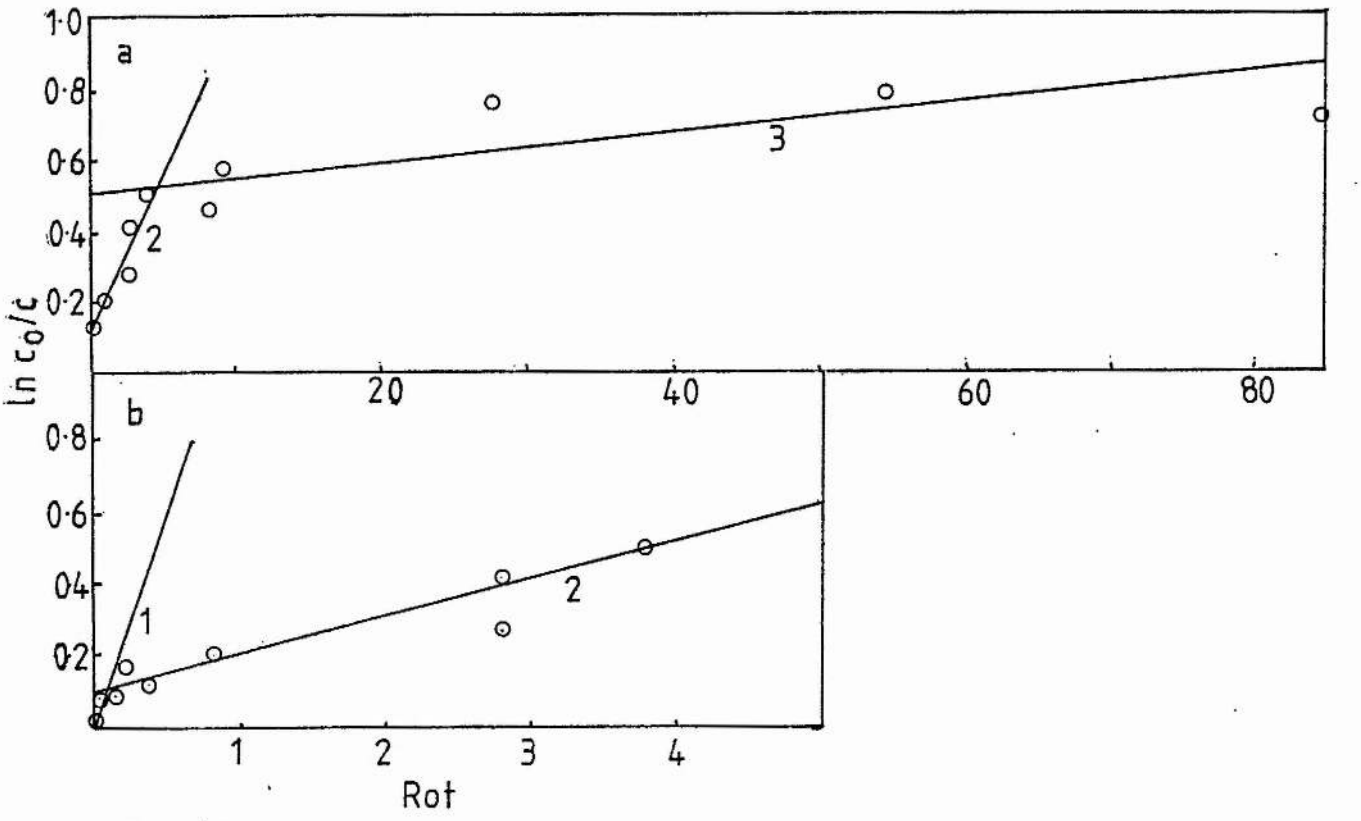

Fig 43 a and b

Linear plot of the data in Fig. 42 The numbers correspond to the transitions denoted in Fig. 42

are scattered. Secondly, in correcting the Rot$\frac{1}{2}$, it has been assumed that the factors affecting it are similar for all frequency classes of RNA - e.g. GC content, poly(A) tract length and average size. These factors are naturally derived from the most frequent RNA component and may not apply to the least frequent class. It must be accepted that these values can vary by a factor of at least two. This may account for the two-fold difference between the values of the percentage of the genome transcribed derived for saturation kinetics and from cDNA-polyA$^+$RNA hybridisation kinetics.

(f) Complexity of Paramecium polyA$^+$RNA from D cells

PolyA$^+$RNA was isolated from cells grown at 32^{o}C and hybridised to its cDNA copy (Fig. 42). Again there are complex hybridisation kinetics. Unfortunately the values are somewhat more scattered than in the previous complexity curve, which makes it difficult to analyse accurately. As before, the hybridisation kinetics have been considered to be a combination of three first-order curves. Plotting the curve as ln Co/C vs Rot gives the amount of cDNA involved in each transition and the Rot$\frac{1}{2}$ value (Fig. 43 a and b). As before the values have been corrected for a number of factors (see Table 6).

The kinetics of the two hybridisation reactions are qualitatively similar, but differ in the exact values of each transition. This could be accounted for in a number of ways, e.g. in the amount of contaminating non-polyA$^+$RNA. Such RNA would not contribute to the hybridisation but would increase the apparent Rot values. Furthermore, as discussed before,

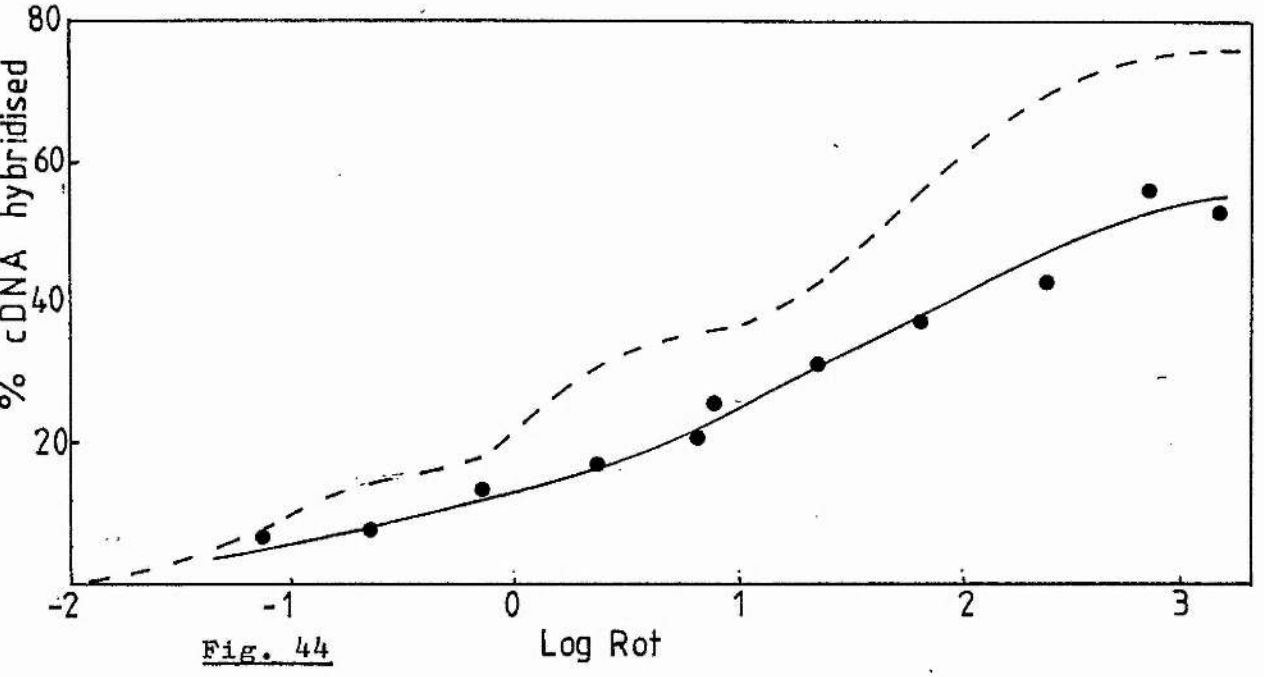

Fig. 44

Heterologous hybridisation of D polyA$^+$RNA with G cDNA
The dotted line indicates the hybridisation of G polyA$^+$RNA with G cDNA.

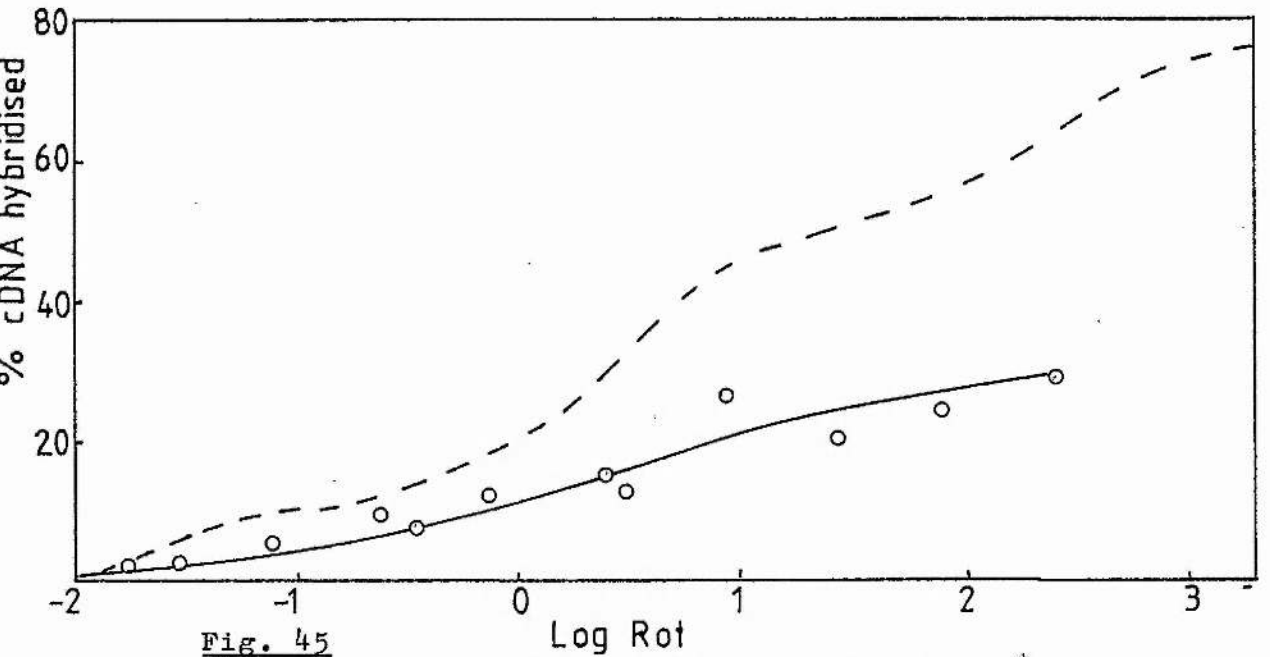

Fig. 45

Heterologous hybridisation of G polyA$^+$RNA with D cDNA
The dotted line indicates the hybridisation of D polyA$^+$RNA with D cDNA

the correction factors used may not apply to the lowest frequency class of RNA. This class however accounts for the majority of the sequence complexity of the RNA and therefore values derived for this transition may be incorrect by a factor of at least two.

Nevertheless there do appear to be differences in the hybridisation kinetics of polyA$^+$RNA isolated from the two types of cell, particularly in the least frequent class.

(g) Heterologous hybridisations

Saturation kinetics suggest that, while some RNA species are common to cells grown at 25^{o}C, some are unique to each cell type. The representation of each RNA frequency class of one cell type in the RNA of the other cell type can be determined by the hybridisation of polyA$^+$RNA with heterologous cDNA.

cDNA prepared to polyA$^+$RNA from G cells when hybridised to polyA$^+$RNA from D cells is only hybridised to a plateau value of 55% (Fig. 44). This is 70% of the plateau value of a homologous hybridisation suggesting that 70% of the sequences are in common, in the two cell types. By saturation this value is estimated to be 55%, but, as discussed previously, this latter is an approximate value.

Comparing the heterologous and homologous hybridisation, it appears that the sequences not present in D polyA$^+$RNA include these which are most frequent in the G cells as well as the least frequent RNA species.

The second hybridisation (Fig. 45) compares the hybridisation of cDNA prepared from D polyA$^+$RNA with polyA$^+$RNA

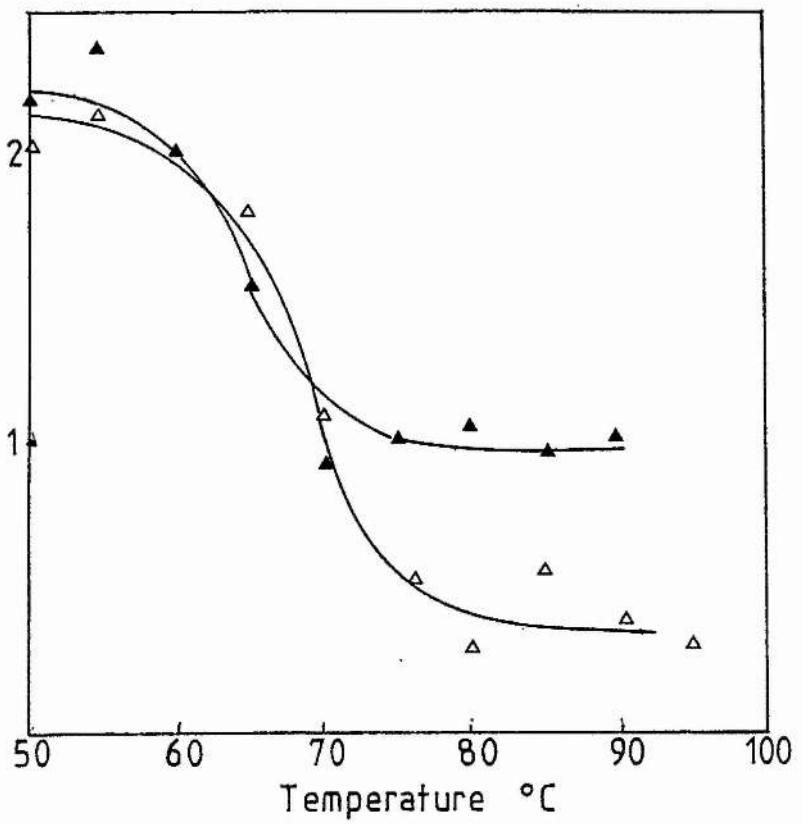

Fig. 46

Melting profile of cDNA - polyA$^+$RNA hybrid compared with the melting profile of ^{125}I DNA-DNA hybrid.

△ - cDNA-polyA$^+$RNA hybrid

▲ - ^{125}I DNA - DNA hybrid

from G cells to the homologous hybridisation of DcDNA and D polyA$^+$RNA. Again not all of the DcDNA is hybridised by G polyA$^+$RNA. Although the Rot value reached by the homologous reaction has not been attained under the conditions used in the heterologous reaction, it can be estimated that only about 40% of the RNA sequences in D cells are also present in G cells, in agreement with the saturation data. Again, like the heterologous G reaction, the sequences not present in G cells include those represented at a high frequency in D cells.

(h) Thermal stability of cDNA - polyA$^+$RNA hybrids

cDNA prepared from G polyA$^+$RNA was hybridised to a Rot value of 1500. The sample was diluted and heated to 90°C by 5°C increments. Samples were taken at every increment and the percentage of cDNA remaining double-stranded assayed by S1-nuclease digestion. Under the conditions used the Tm was 68.5°C (Fig. 46). This is equivalent to a Tm of 81°C in 1SSC. Under similar conditions an iodinated DNA - DNA hybrid had a Tm equivalent to 79°C. The 2°C difference could be due to the polyA$^+$RNA, and thus the cDNA, having a slightly higher GC content than the bulk of the DNA. Hruby et al. (1977) estimated the GC content of polyA$^+$RNA from Paramecium to be 35%, 10% higher than the GC content of the DNA.

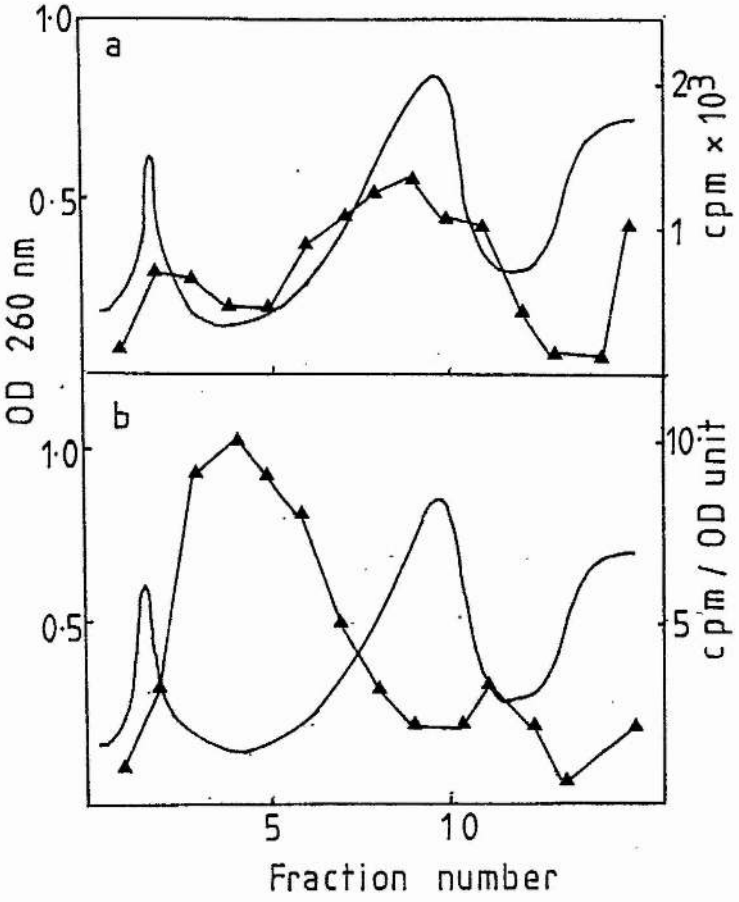

Fig. 47

Gradient analysis of PMS from cells labelled with uridine labelled bacteria for 2 hours.

a) ▲ - cpm/fraction b) ▲ - cpm/OD unit

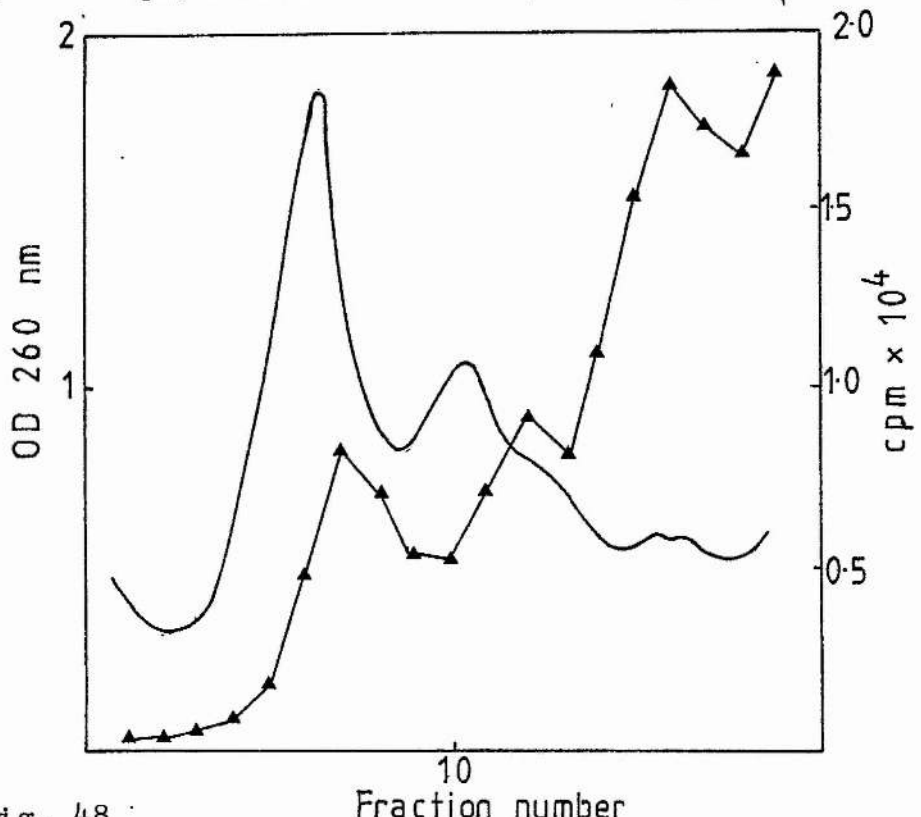

Fig. 48

Gradient analysis of RNA from the PMS of cells labelled with uridine labelled bacteria for 2 hours

▲——▲ - c.p.m.

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(iii) RNA Synthesis

(a) Radioactive labelling of Paramecium by supplying labelled bacteria

RNA synthesis was examined initially by growing paramecia in the presence of radioactively labelled bacteria and allowing the paramecia to digest the bacteria and utilise the labelled metabolites. The paramecia were then washed free of exogenous bacteria and, by gradient analysis, the size distribution of labelled RNA examined.

1) Distribution of label in polysomal fractions

Post-mitochondrial supernatant (PMS) was made from cells which had been labelled for 2 hours with tritiated uridine labelled bacteria. Fig. 47 a shows the distribution of label on a sucrose gradient. It can be seen that labelled uridine has been incorporated into various sizes of particle and particularly into the monosome peak, but there is higher specific activity in the polysomal region (Fig.47 b).

2) Distribution of label in RNA size classes

RNA was extracted from the PMS and examined by sucrose gradient analysis. Fig. 48 shows that there is considerable labelling of the 4/5S region, but that there is also label in material smaller than 18S at approximately 12S. This may correspond to the 18S breakdown product. There is also incorporation into material slightly smaller than 25S. The 25S and 18S ribosomal RNAs do not appear to be well labelled after 2 hours.

3) The effect of labelling with bateria for different lengths of time.

Paramecia were incubated as a 1% suspension in labelled

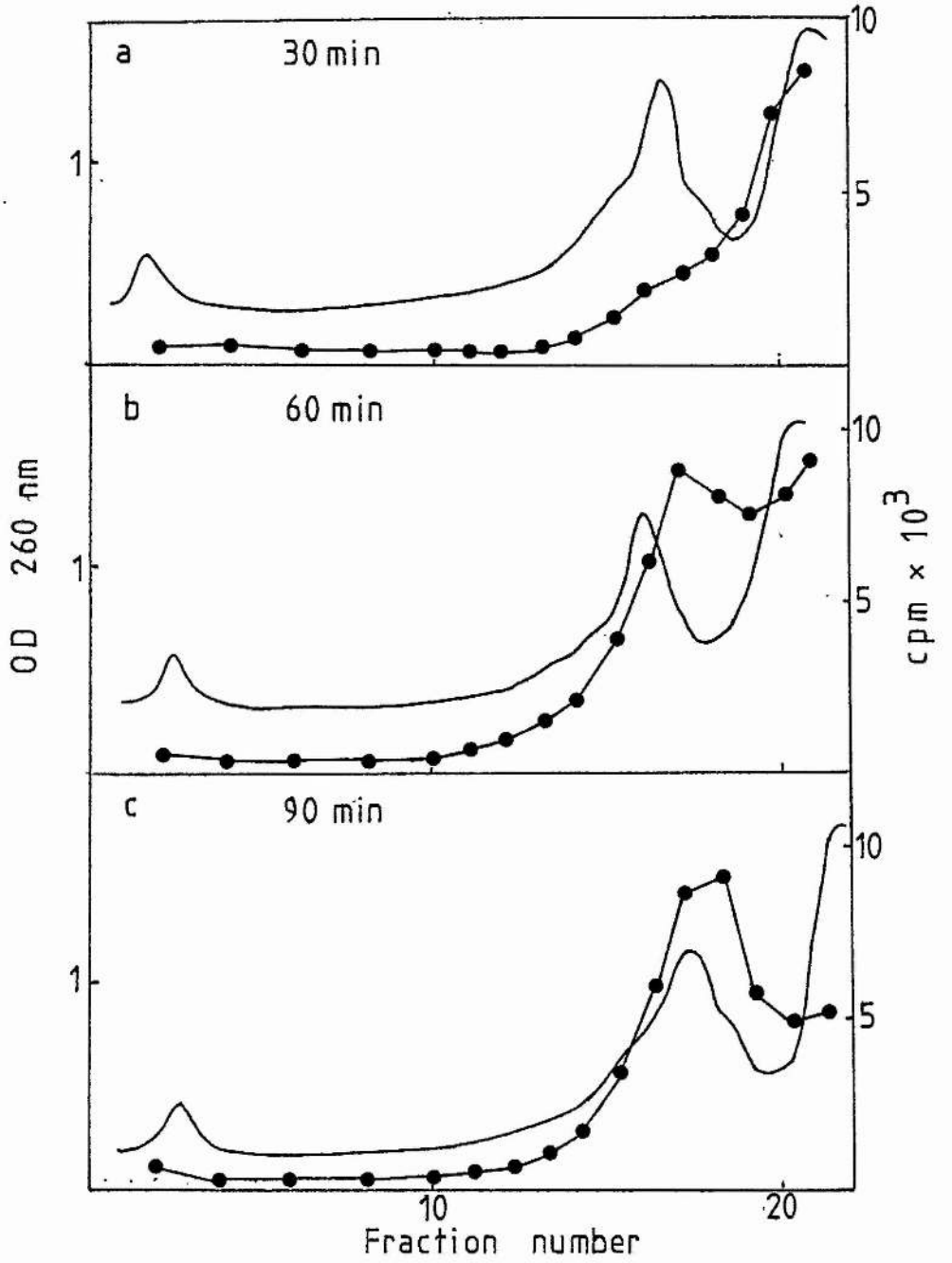

Fig. 49

Gradient analysis of PMS from cells labelled with uridine labelled bacteria for different lengths of time.

a) 30 minutes
b) 60 minutes
c) 90 minutes

●—● - c.p.m.

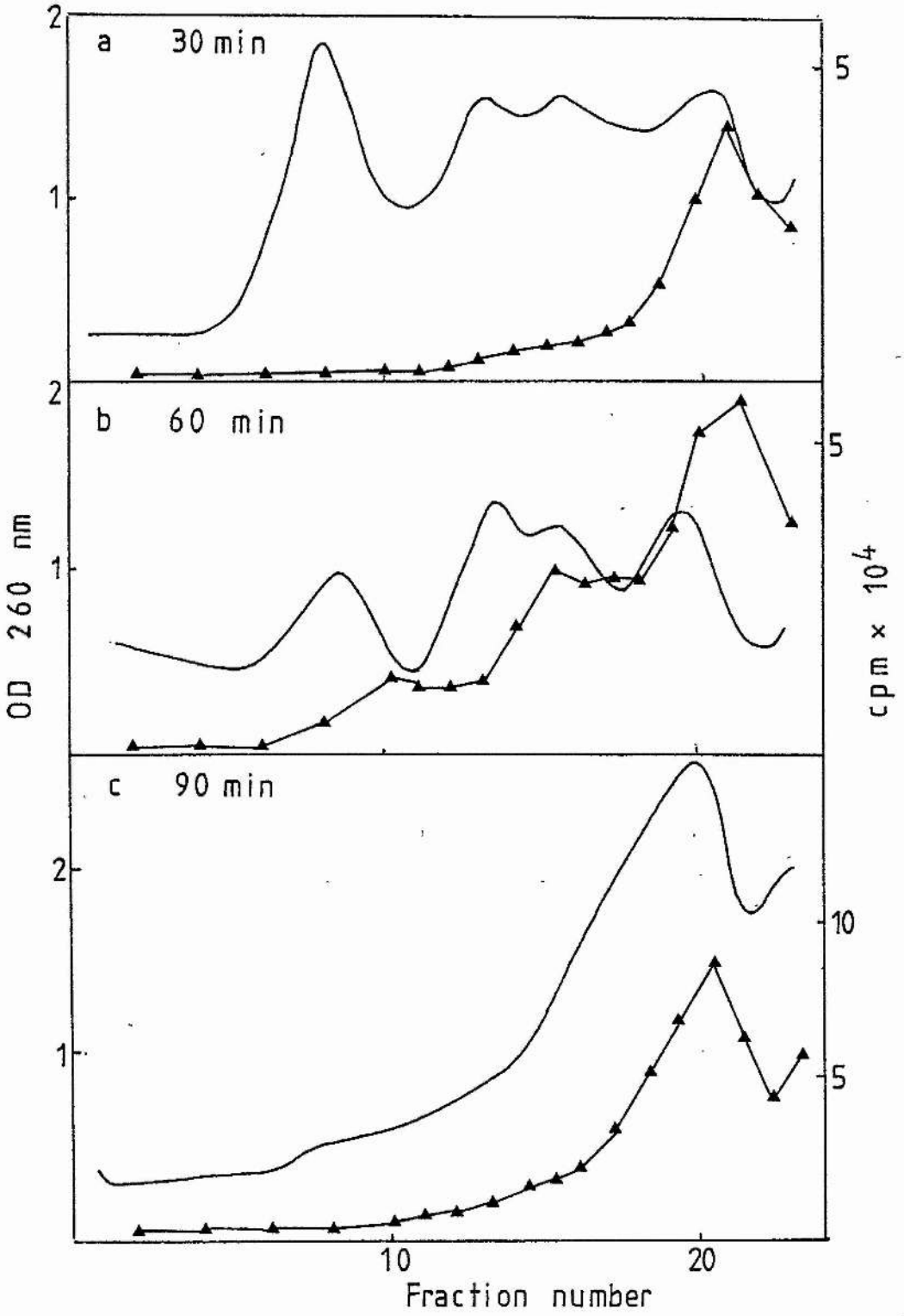

Fig. 50

Gradient analysis of RNA isolated from the PMS of cells labelled for 30, 60 and 90 minutes with uridine-labelled bacteria.

▲—▲ cpm.

bacteria for 30, 60 and 90 minutes before being washed and harvested. PMS was prepared from each sample of cells and analysed by sucrose gradient centrifugation (Fig.49 a, b and c). There is progressive incorporation into larger material, but little incorporation into the polysome region. Incorporation appears to be mainly into material smaller than 80S and only by 90 minutes in there significant incorporation into monosomes. The optical profile indicates that there are very few polysomes. The incubation conditions of high cell density may cause degradation of polysomes and the release of newly labelled mRNAs as mRNPs which would generally be smaller than 80S.

RNA was prepared from the PMS and analysed by sucrose gradient centrifugation. Fig.50a, b and c shows that there is some breakdown of RNA occurring and that, by 90 minutes, this is extensive. In all three gradients the majority of labelled material is less than 4S in size. By 60 minutes there is some incorporation into material between 4 and 18S in size, but this may be 18S breakdown products rather than mRNA. There is a further peak of labelling at approximately 20S.

The pattern obtained after labelling for 60 minutes is similar to the pattern obtained after labelling for 2 hours, as described earlier. There is clearly a problem with RNA breakdown during the incubation conditions. As described in section 1 of this Chapter, the preparation of RNA from PMS seems to encourage RNA breakdown, particularly of 18S RNA.

4) Adenosine labelling of paramecia

Paramecia were similarly labelled for 30, 60 and 90 minutes using bacteria which had been labelled with adenosine.

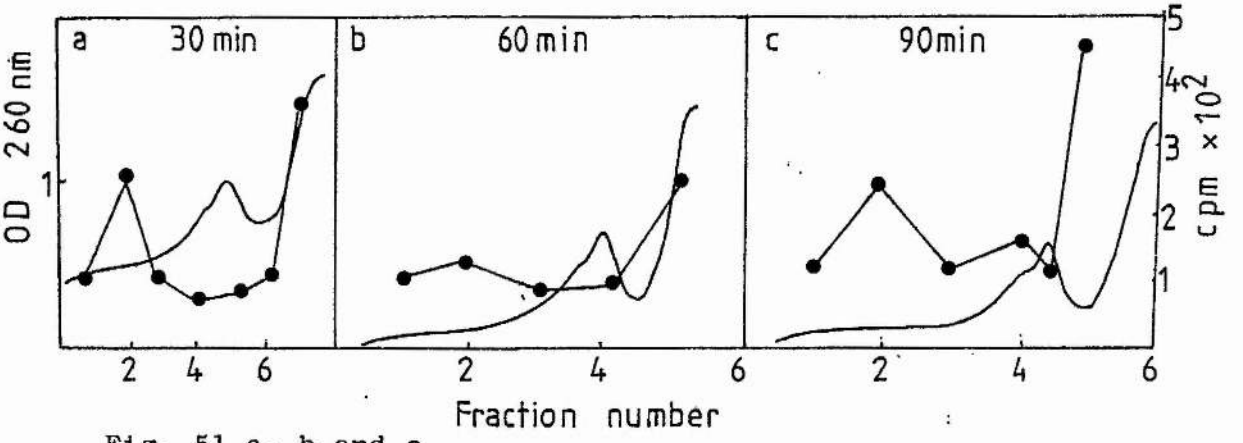

Fig. 51 a, b and c
Gradient analysis of PMS from cells labelled with adenosine labelled bacteria for different lengths of time.

a) 30 minutes b) 60 minutes c) 90 minutes

●—● cpm

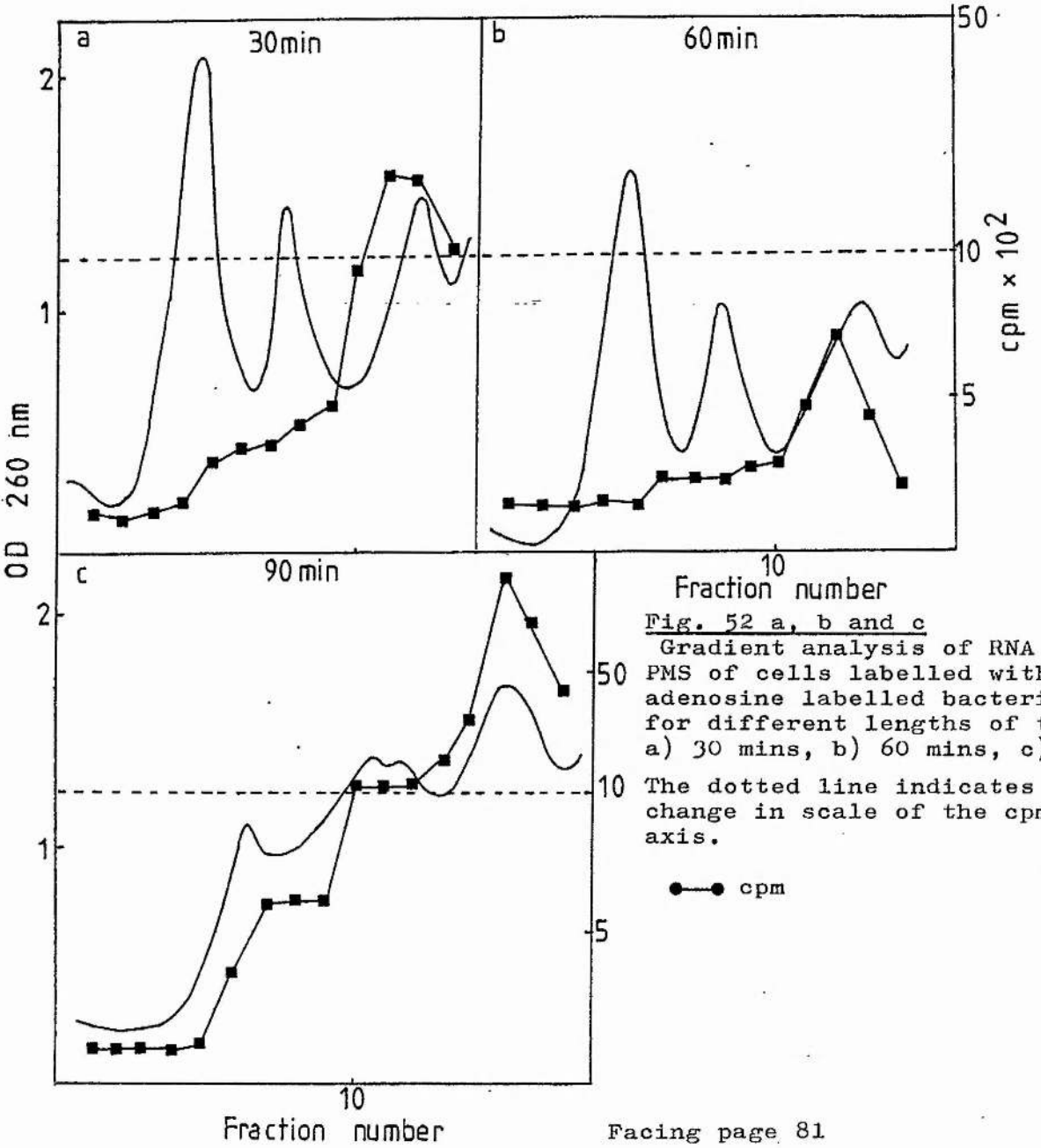

Fig. 52 a, b and c
Gradient analysis of RNA from PMS of cells labelled with adenosine labelled bacteria for different lengths of time a) 30 mins, b) 60 mins, c) 90 mins.
The dotted line indicates a change in scale of the cpm axis.

●—● cpm

The polysome profile is similar to that of uridine labelled cells, there being little incorporation into monsomes until 90 minutes (Fig. 51 a, b and c). The RNA profile again shows that there is some breakdown of RNA after 90 minutes, although it is less extensive in this experiment. (Fig, 52 a, b and c). As in uridine labelled cells, the majority of labelling is of small components, although, in this case, larger than 4S, which may be a feature of the apparent lower RNA breakdown in this preparation. There is very little labelling of 25 and 18S RNA until 90 minutes, where the labelling pattern is obscured by the extensive labelling of the 4S peak.

Although this method of labelling has been used successfully to label proteins with radioactive amino-acids (Sommerville, 1968) or, using lower cell densities, to label DNA (Berger and Kimball, 1964) and the poly(A) tract of polyA$^+$RNA, it is clearly inadequate for the short-term labelling of RNA for a number of reasons. Firstly, high cell densities appear to be disruptive to cell metabolism. Prolonged incubation of cells as a 1% cell suspension causes cell death. Polysomes and RNA appear to be particularly sensitive to the effect of high cell density, possibly due to the disruption of lysosomes which would release nucleases. The use of 0.5% cell suspensions reduced, but did not entirely remove, this effect. The high bacterial density, necessary to obtain reasonable levels of incorporation in short times, may enhance the activity of lysosomes since these are involved in the digestion of ingested bacteria. Secondly the preparation of PMS is necessary since it removes any intact bacteria by centrifugation. However, as

indicated in section i of this Chapter, there appears to be some nuclease activity inherent in this preparation. Thirdly, although this preparation removes intact bacteria, partially degraded bacteria may not be removed and could contribute to labelled material in the preparation. Bacterial RNA will be labelled to a much higher specific activity than will Paramecium RNA and so a small amount of bacterial RNA will contribute considerably to the labelling pattern. It is likely that the extensive labelling of the 4S region is due to partially digested bacterial RNA. In time this will decrease as bacteria are completely digested and the metabolites used in synthesis of Paramecium RNA. However this may take in excess of 2 hours since the life-span of a food-vacuole is approximately 2 hours in the conditions of incubation employed (Berger and Kimball, 1964).

In view of these problems an alternative method of labelling was used. In this method, cells were washed free of bacteria and resuspended as a 0.5% suspension in MS which contained 10mM sodium formate to minimise the utilisation of scavenged labelled products (Cummings, et al., 1974). This is similar to the method used by Cummings (1975) to label ribosomal RNA, although here MS rather than a citrate buffer (Dryl, 1959) and shorter labelling times have been used.

5) Comparison of labelling methods

Paramecia were labelled as a 0.5% suspension for 2 hours in either MS containing 2 mg/ml bacteria labelled overnight with 5μCi/ml uridine (27 Ci/ mmole) or MS containing 1μCi/ml uridine

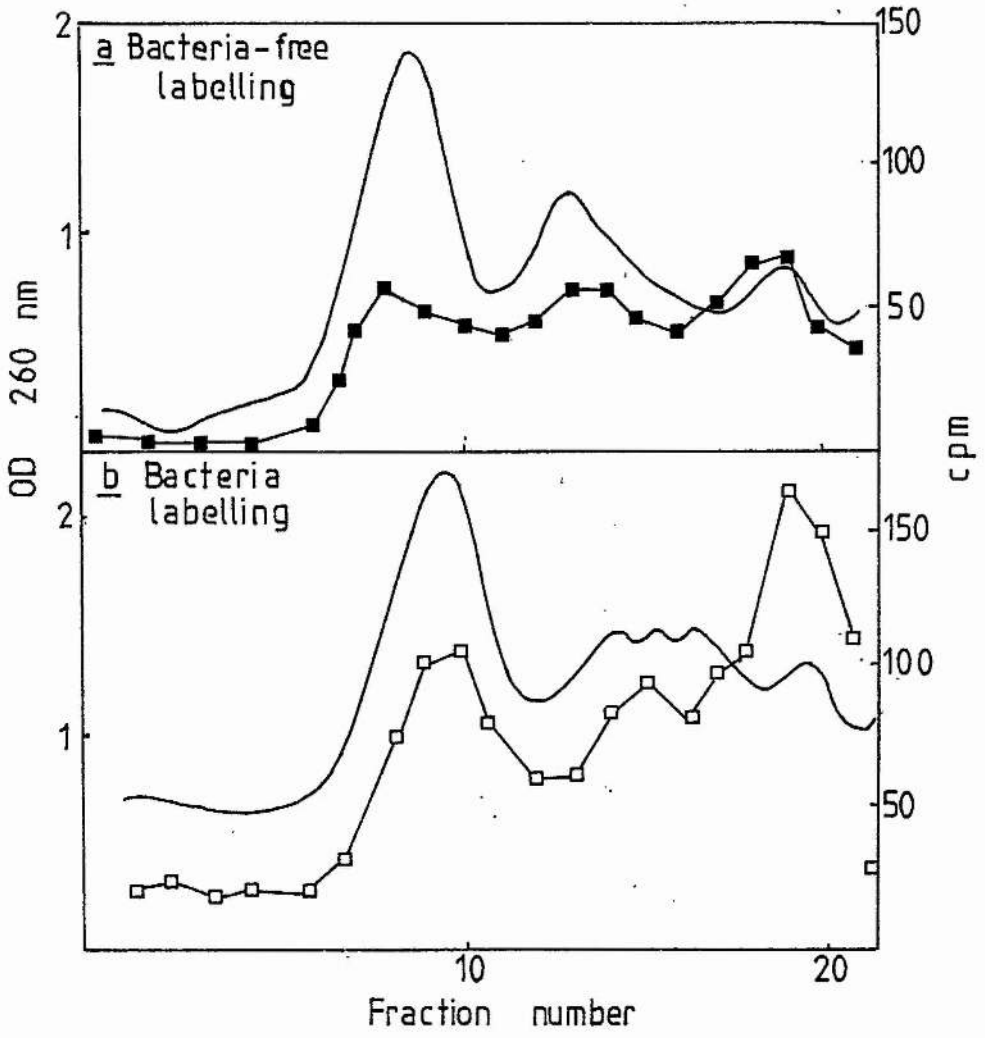

Fig. 53 a and b

Gradient analysis of RNA labelled a) with bacteria and b) under bacteria-free conditions.

a) ■ Bacteria-free labelling

b) □ Bacteria labelling

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(27 Ci/m mole). At the end of the labelling period the cells were washed and transfered to sterile grass medium for 2 hours.

The RNA from bacteria-labelled cells had incorporated 4 times as much label as RNA from bacteria-free labelled cells. An examination of RNA from the two cell samples by sucrose gradient analysis, shows a difference in labelling pattern (Fig. 53 a and b). Although the bacteria-labelled cells have incorporated more radioactivity, much of it is in the 4S peak, whereas in the RNA from cells labelled under bacteria-free conditions, there is much less radioactivity in the 4S peak. The remaining labelled material is in the 25S and 18S peaks with some labelling of material smaller than 18S. Although the specific activity is lower, the bacteria-free labelling method appears to label Paramecium RNA more representatively than does the bacterial method and also avoids the problem of possible contamination with bacterial RNA. However, it must be borne in mind that the labelling of cells in non-nutrient medium is not ideal since there may be metabolic changes associated with the withdrawal of food. On the other hand, paramecia will stay active for several days in MS and it is likely that any effect due to culture in MS would be neither extensive nor immediate. The serotype of the cells, for example, remains stable on transfer to MS (see Chapter IV).

Labelling in MS also has the advantage that RNA can be extracted from intact cells rather than from the PMS since there should be no labelled bacteria present. Routinely cells were washed extensively with MS and incubated as a 0.5% suspension

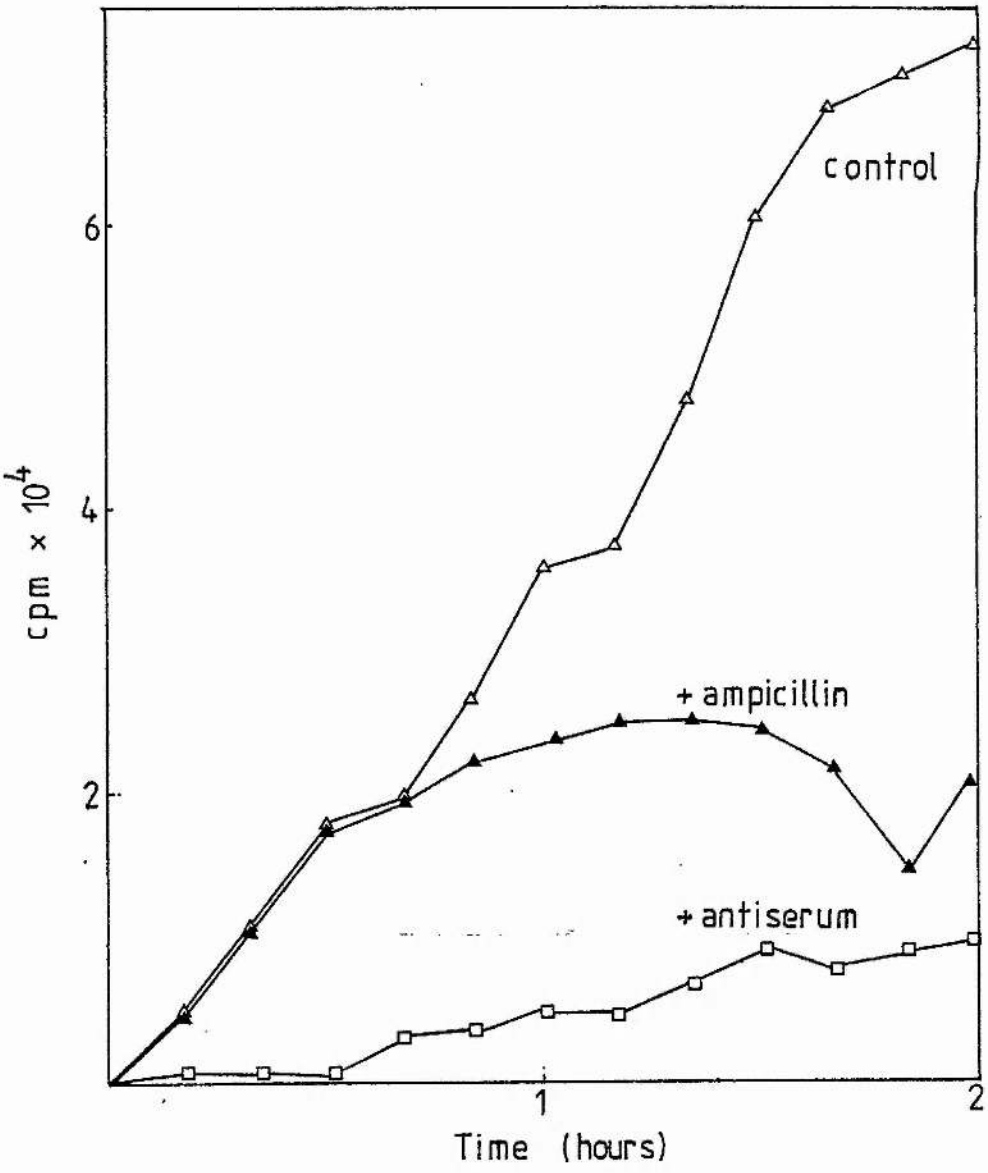

Fig. 54

Labelling of cells with uridine in bacteria-free conditions in the presence of ampicillin or homologous antiserum. The cells were incubated with either ampicillin or antiserum for 2 hours prior to the addition of uridine, and incubation continued for a further 2 hours during which time samples were taken.

△ - control
▲ - + ampicillin 10 µg/ml
□ - + antiserum

for 15-30 minutes prior to labelling with uridine to remove most of the remaining bacteria. An examination of the culture medium at this stage showed very few, if any bacteria. After labelling, cold uridine was added and the cells immediately harvested and homogenised in Kirbys buffer. The time from addition of cold uridine to homogenisation was generally 4 minutes and can be regarded as a 4 minute cold chase.

(b) Bacteria-free labelling of paramecia with uridine

Although no bacteria could be seen in a suspension of well washed cells, since bacteria label very efficiently in comparison to paramecia, it was essential to demonstrate that any incorporation of uridine into TCA precipitable material was due to paramecia rather than to the trace amount of bacteria which might be present. Accordingly, washed cells were resuspended as a 0.5% suspension in MS and the suspension divided into three. One sample was treated with ampicillin at a concentration sufficient to cause lysis of any Klebsiella aerogenes present (10 µg/ml). A second sample was treated with antiserum, sufficient to immobilise at least 80% of the paramecia. The remaining sample was left untreated. Samples were incubated for 90 minutes prior to the addition of uridine and samples taken at intervals for 2 hours. Fig.54 shows the effects of ampicillin and antiserum. In the control flask incorporation remains linear for two hours. In the flask treated with ampicillin, incorporation is initially at the same rate as that of the control flask but tails off after 30 minutes of labelling i.e. 2 hours after incubation of the

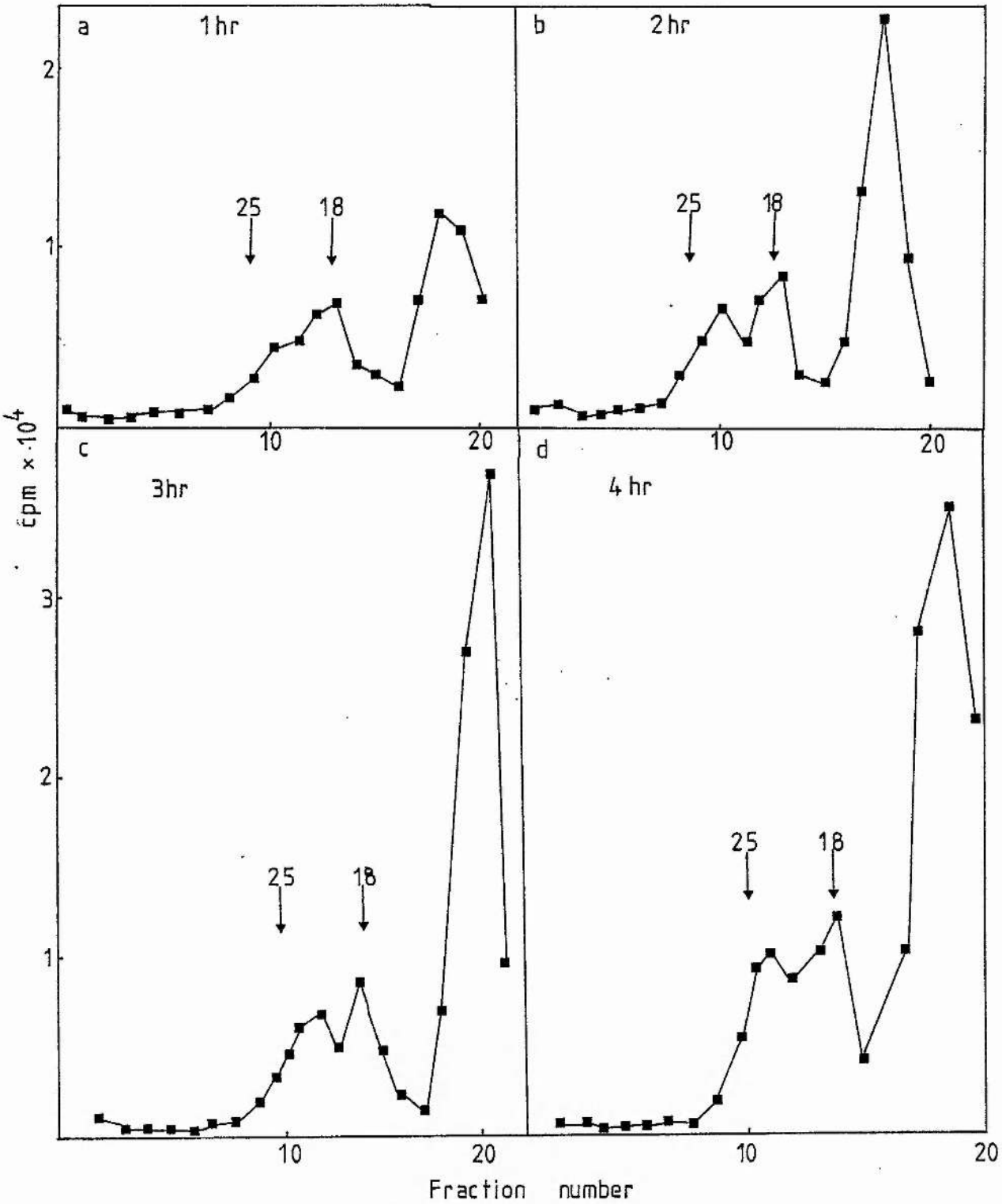

Fig. 55 a, b, c and d

Gradient analysis of RNA from cells labelled under bacteria-free conditions at 25°C for a) 1 hour, b) 2 hours, c) 3 hours and d) 4 hours.

The positions of the optical peaks of 25 and 18S ribosomal RNA are indicated by arrows

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paramecia with ampicillin. This may indicate some late acting effect of either ampicillin or lysed bacteria on the paramecia. In the flask treated with antiserum, incorporation is at a rate 14% of that of the control flask. This level of incorporation can be attributed to the 20% non-immobilised cells. Were the incorporation in the control flask due significantly to bacteria, then the level of incorporation in the antiserum-treated flask would be expected to be higher than 20%. Thus, uridine incorporation under the conditions used can be assumed to be due to the paramecia .

2) Incorporation of uridine after 1-4 hours of labelling

After 1 hour of labelling with uridine 18S RNA is labelled, together with the 4S peak Fig.(55 a, b and c). There is proportionately little labelling of the 25S peak but, as labelling continues, the 25S peak becomes progressively more labelled. The 18S and 25S peaks however do not become labelled in proportion to the optical amounts of the two ribosomal RNAs, even after 4 hours. Although the 18S peak of labelling corresponds closely to the optical 18S peak, the higher S-value peak of labelling is consistently smaller than the 25S optical peak and falls within the range 20-22S (see Fig.55). This is not due to some anomaly in fraction collecting, since, if one measures the OD of each fraction after collection, the value corresponds closely to the optical trace (Fig.56). It therefore appears that an RNA species approximately 20-22S in size is being synthesised in large amounts and obscuring the 25S peak.

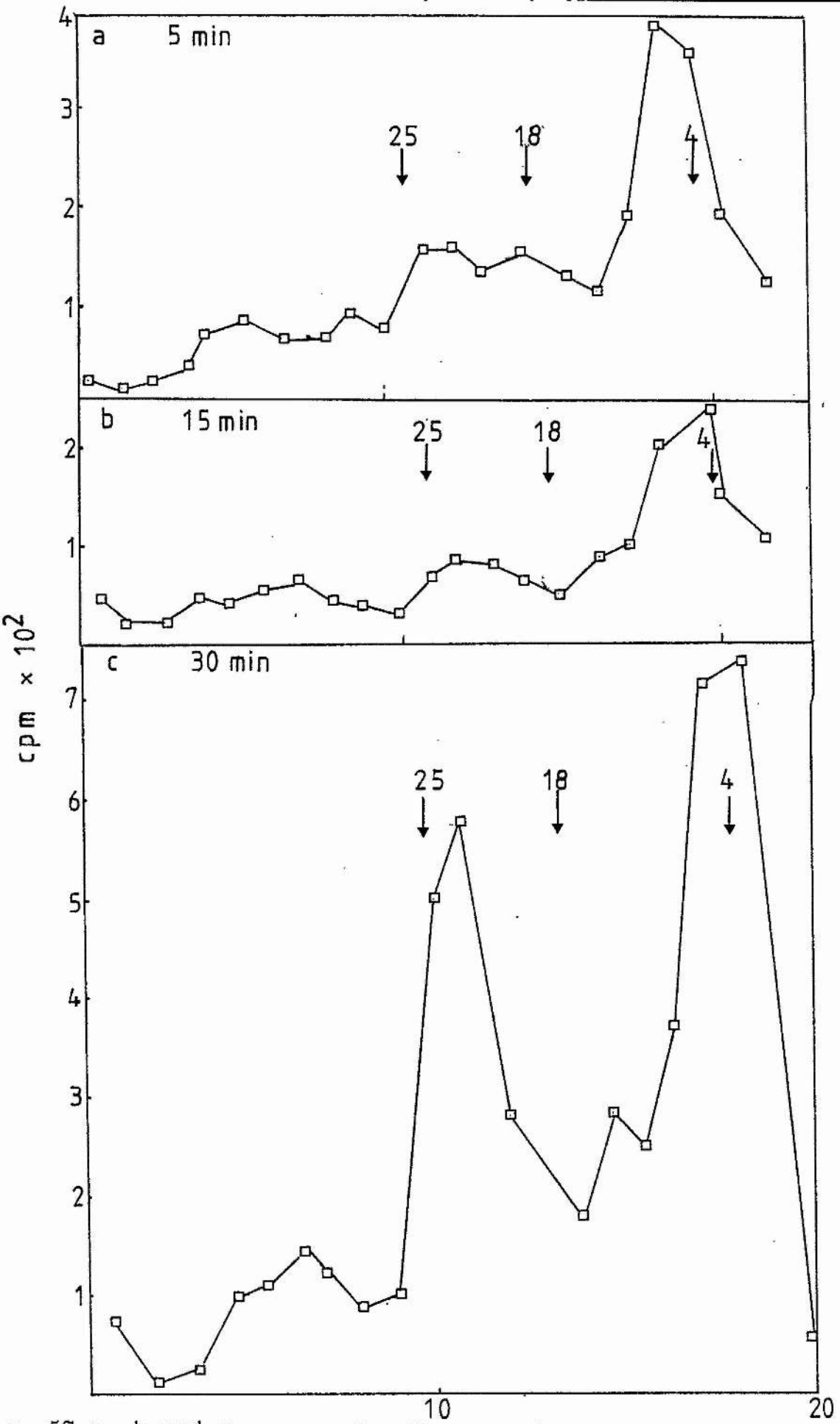

Fig. 57 a, b and c

Gradient analysis of RNA from cells labelled under bacteria-free conditions at 25°C for a) 5 mins, b) 15 mins and c) 30 mins. The positions of the optical peaks 25 and 18S and 4/5S are indicated by arrows.

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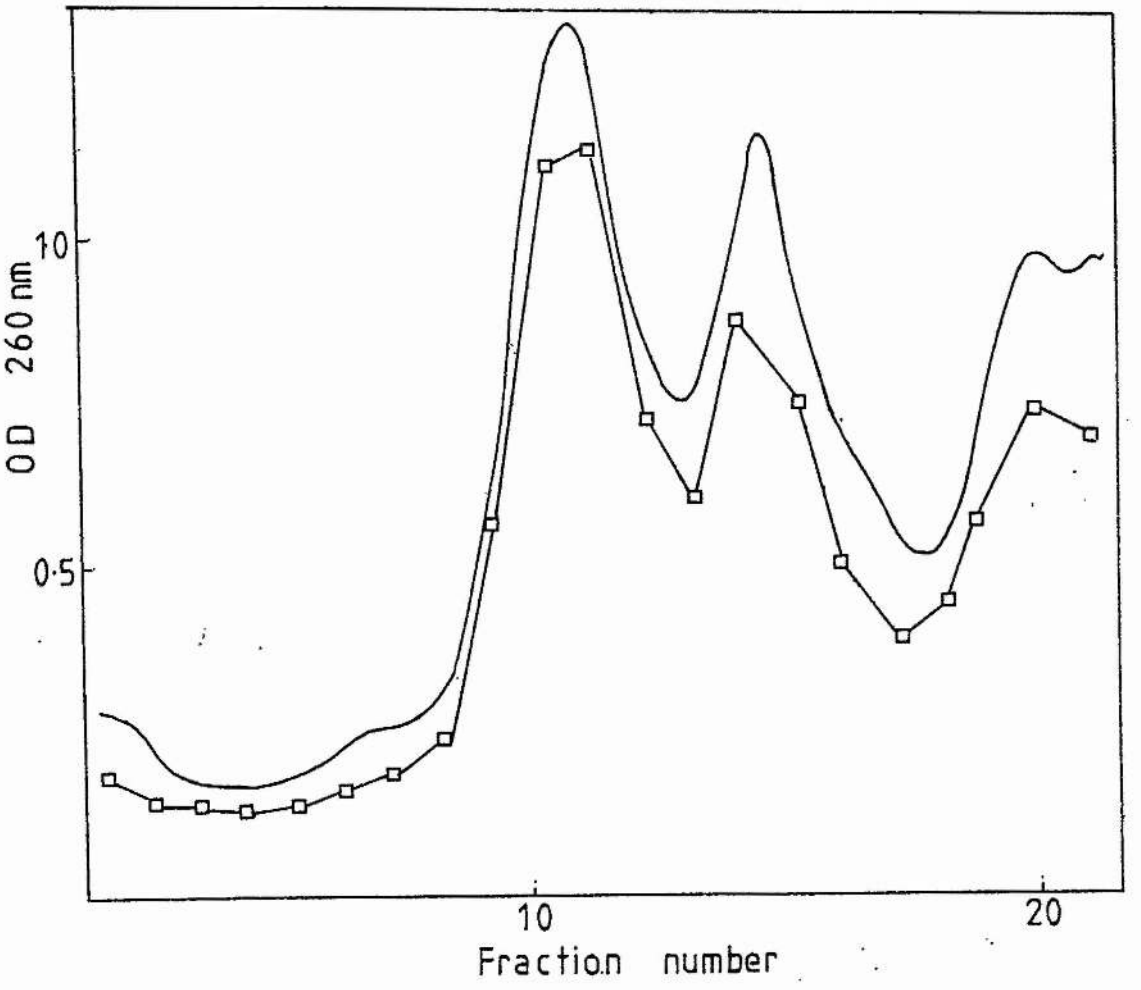

Fig. 56

A comparison between the optical trace of a gradient of RNA and the OD of collected fractions.

□ - OD of each fraction

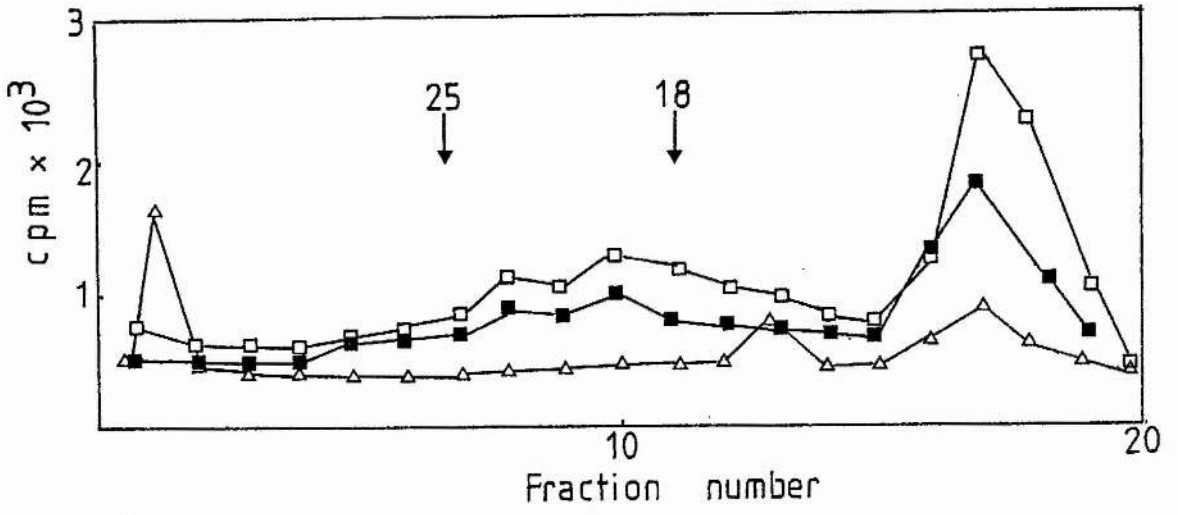

Fig. 58

Gradient analysis of RNA from cells labelled under bacteria-free conditions at 25°C for 5 minutes, 15 minutes and 30 mins. The position of the optical peaks of 25S and 18S ribosomal RNA are indicated by arrows.

△ - 5 min. ■ - 15 min. □ - 30 mins.

There is little labelling of the region between 4S and 18S, the area in which one might expect to find most of the rapidly labelled mRNAs. Incorporation at 25°C was, however, too low to allow an examination of the distribution of label in either polyA$^+$RNA or polysomes. Another feature of this labelling procedure is the lack of consistent labelling of the 34S region, the putative ribosomal RNA precursor (Prescott et al., 1971)

3) Short term labelling with uridine

Cells were labelled under similar conditions for 5, 15 and 30 minutes (Fig. 57 a, b and c). Again the 4/5S region is highly labelled. The 4/5S peak of labelling is initially approximately 8S in size and becomes progressively smaller as labelling proceeds. This may indicate that RNA species larger than 4S are being synthesised and that these are gradually obscured by the stable 4S species. The remainder of the labelled RNA is heterogenous in size with peaks in the 34S region and 22S, clearly seen as two separate peaks after 30 minutes (see Fig. 57 c). These peaks are however, not always observed. In a similar experiment a more heterogenous size distribution of labelled RNA is seen (Fig. 58).

4) Labelling of paramecia grown at 32°C with uridine

Labelled RNA from cells grown at 32°C shows a similar pattern of labelling to that of RNA from cells grown at 25°C (Fig.59a, b and c). Again there is considerable incorporation into the 4/5S peak and, as before, becoming progressively smaller. The second peak of labelling is initially between the

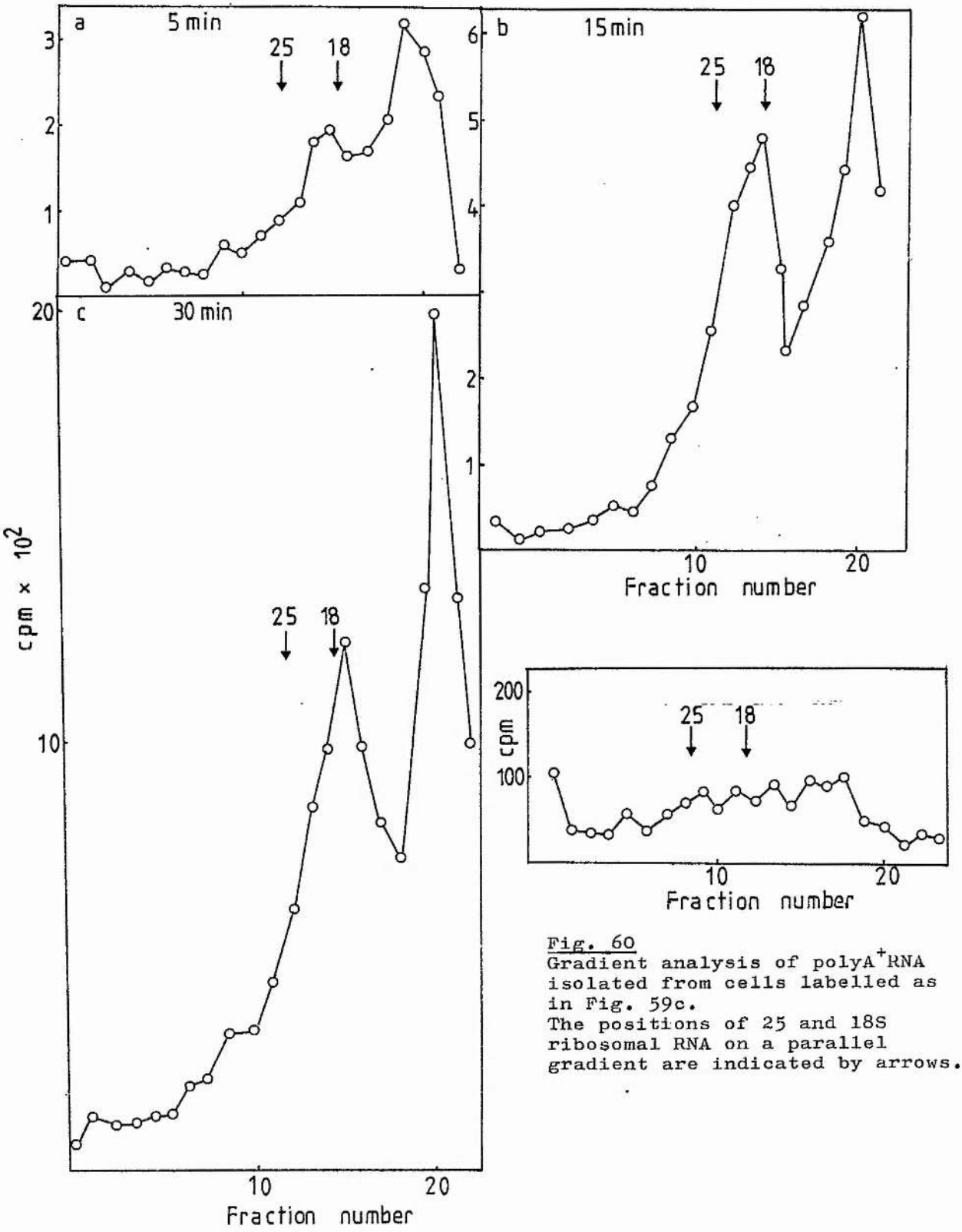

Fig. 60
Gradient analysis of polyA$^+$RNA isolated from cells labelled as in Fig. 59c.
The positions of 25 and 18S ribosomal RNA on a parallel gradient are indicated by arrows.

Fig. 59 a, b and c
Gradient analysis of RNA from cells labelled under bacteria-free conditions at 32°C for a) 5 minutes, b) 15 minutes, c) 30 minutes.
The positions of the optical peaks of 25 and 18S ribosomal RNA are indicated by arrows

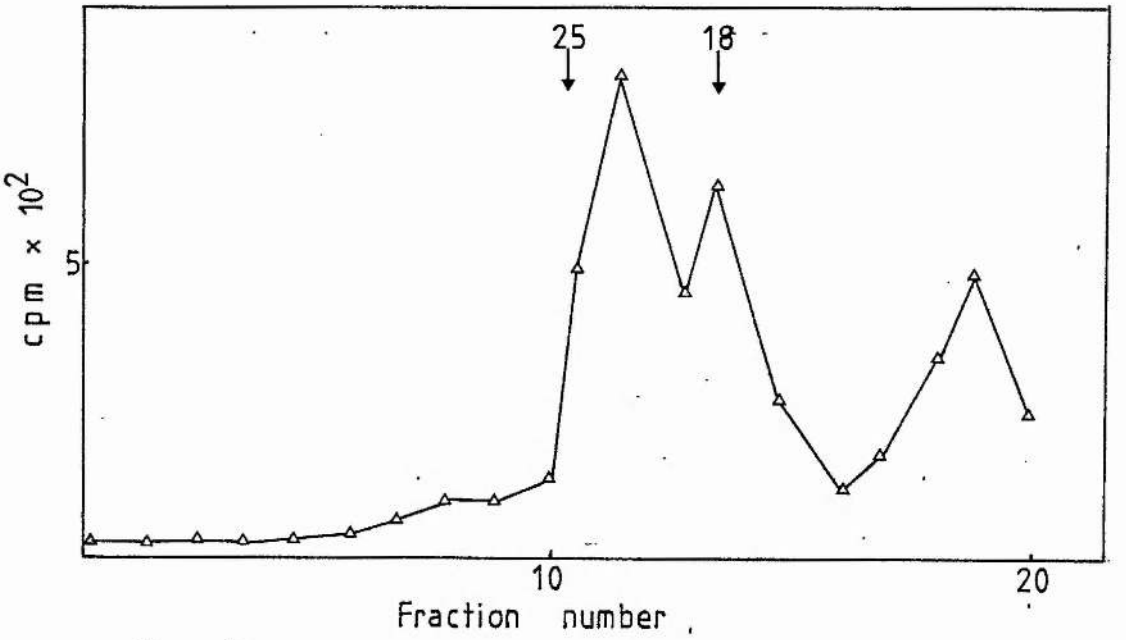

Fig. 61

Gradient analysis of RNA from cells labelled under bacteria-free conditions at 32°C for 30 minutes, followed by a 15 minute cold chase of uridine.

The positions of the optical peaks 25 and 18S ribosomal RNA are indicated by arrows.

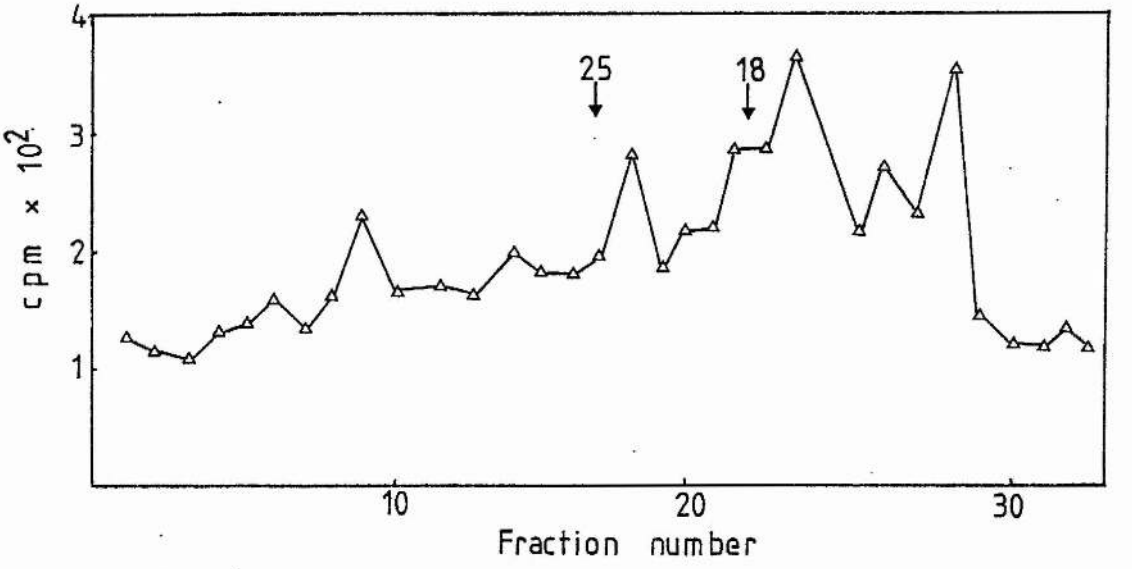

Fig. 62

Gradient analysis of the polyA$^+$RNA from cells labelled as in Fig. 61.

The positions of 25 and 18S RNA on a parallel gradient are indicated by arrows.

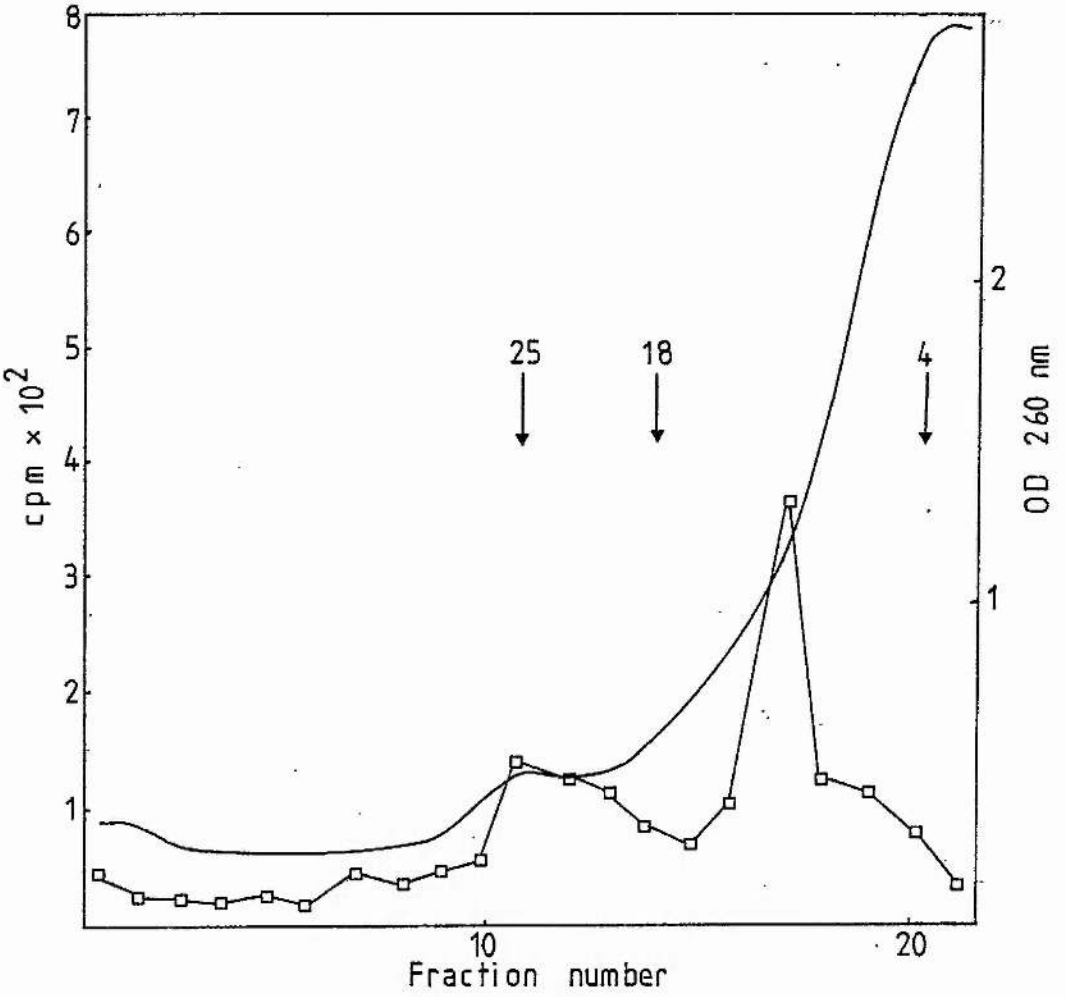

Fig. 63

Gradient analysis of RNA isolated from the macronuclei of cells which had been labelled at 25°C in bacteria-free conditions.

The position of 25 and 18S ribosomal RNA and 4/5S RNA on a parallel gradient are indicated by arrows.

□ - cpm

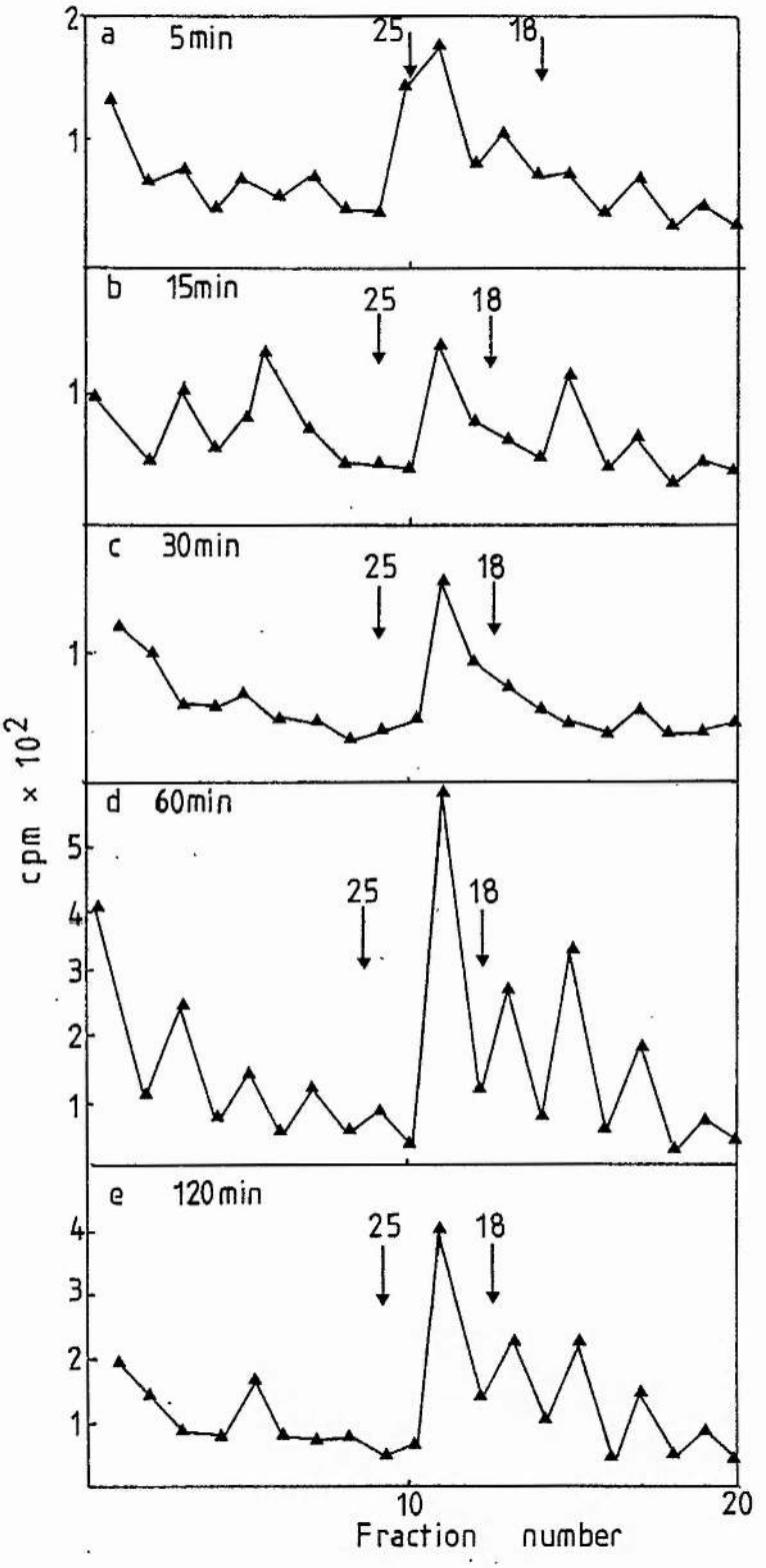

Fig. 64 a, b, c, d and e

Gradient analysis of RNA from cells labelled at 25°C under bacteria-free conditions with ^{3}H-methionine for a) 5 mins, b) 15 minutes, c) 30 minutes, d) 60 minutes and e) 120 minutes. The position of the optical peaks 25and 18S ribosomal RNA are indicated by arrows.

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25 and 18S species but, as labelling continues, becomes smaller and is probably the 18S species. PolyA$^+$RNA isolated from cells labelled for 30 minutes shows a heterogenous size distribution (Fig. 60). Labelling cells for 30 minutes followed by a 15 minute cold chase of uridine shows a differant pattern of labelling with more incorporation into the 20S peak than the 18S peak (Fig. 61). PolyA$^+$RNA isolated from cells labelled in this way again shows a heterogenous distribution, the major peak being at 12S although two peaks are seen at 34S and 20S (Fig. 62).

5) Labelling of nuclear RNA

Nuclei were isolated from cells labelled for 30 minutes with uridine and the RNA isolated and analysed on a sucrose gradient. The optical profile is as found previously with considerable amounts of material near the top of the gradient, obscuring the 18S peak. There is only marginal labelling of the 34S region, and the major peaks of labelling are at 25S and 10S. (Fig. 63).

6) Labelling of cells with tritiated methionine

Paramecia were labelled with tritiated methionine which is used by the cell to methylate RNA and as such can be used to detect ribosomal RNA synthesis since ribosomal RNA is highly methylated. This technique has been used to detect ribosomal RNA synthesis in Tetrahymena (Prescott et al., 1971). Fig. 64 a, b, c, d and e shows a somewhat heterogenous distribution of labelling, the only consistent peak being that of 20S. There is little consistent labelling of the 25, 18 and 4S peaks.

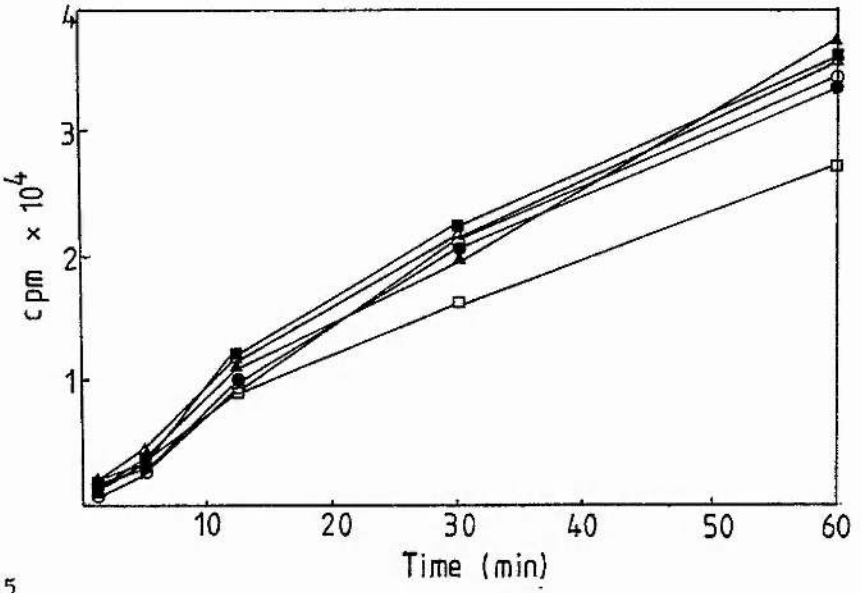

Fig. 65

Incorporation of uridine into TCA precipitable material under bacteria-free conditions in the presence of various concentrations of actinomycin D.

△ - 0 µg/ml ▲ - 0.2 µg/ml ■ - 0.5 µg/ml
□ - 1 µg/ml ○ - 2 µg/ml ● - 5 µg/ml

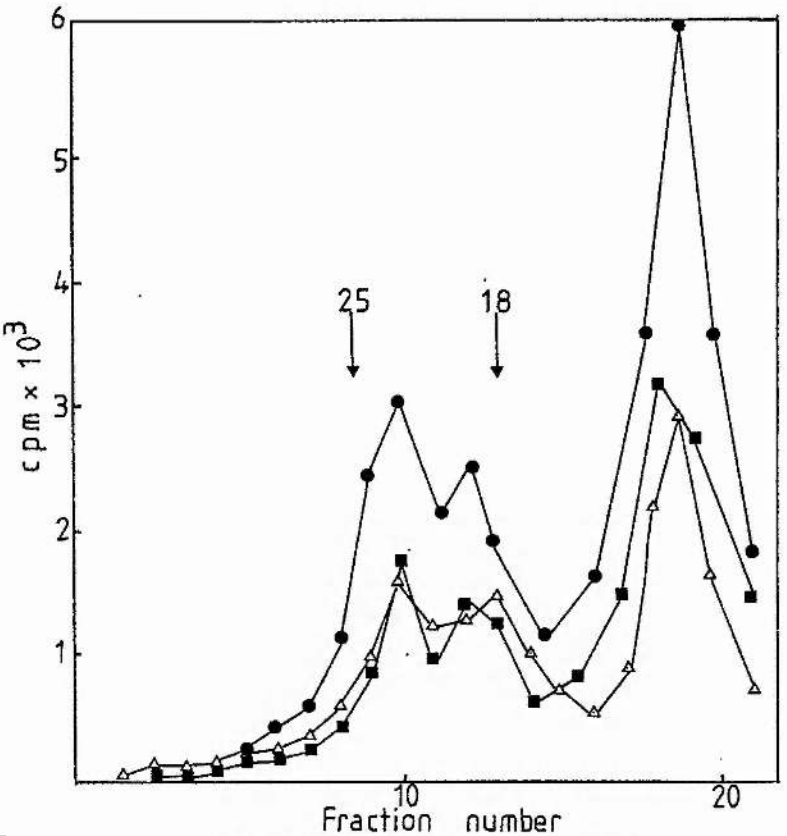

Fig. 66

Gradient analysis of RNA from cells labelled for 30 minutes with uridine under bacteria-free labelling conditions in the presence of actinomycin D.

△ - 0 µg/ml ■ - 1 µg/ml ● - 5 µg/ml

The optical peaks of 25 and 18S RNA are indicated by arrows

7) Labelling of paramecia in the presence of metabolic inhibitors

In order to further investigate the synthesis of ribosomal RNA, cells were labelled with uridine for 1 hour in the presence of actinomycin D which at 0.5 µg/ml is known to inhibit eucayotic RNA polymerase I activity and thus ribosomal RNA synthesis (e.g. Moore and Ringertz, 1973). Cells treated with a low concentration of actinomycin D should therefore synthesise largely mRNA since RNA polymerase II is only inhibited by concentrations of actinomycin D much higher than 0.5 µg/ml. It has been established that growth and division of Paramecium can be inhibited by high doses of actinomycin D (Austin et al., 1967, Sommerville, 1969) but the concentrations required to inhibit the two polymerases of Paramecium are not known, if indeed the RNA polymerases repond to actinomycin D in the same way as those of higher eucaryotes. Accordingly, a range of actinomycin D concentrations were used in order to find optimum conditions for the inhibition of ribosomal RNA synthesis. Fig. 65 shows that, under the conditions of labelling used, actinomycin D generally had no effect on uridine incorporation. An examination of RNA isolated from cells labelled in the presence of 1 and 5 µg/ml actinomycin D, compared with RNA from cells labelled in the absence of actinomycin D shows that this inhibitor has no effect on the pattern of labelling (Fig. 66). As previously found, for cells grown at 25°C and labelled with uridine for 1 hour, radioactivity is found in the 4/5S peak, the 18S and the 20S peak.

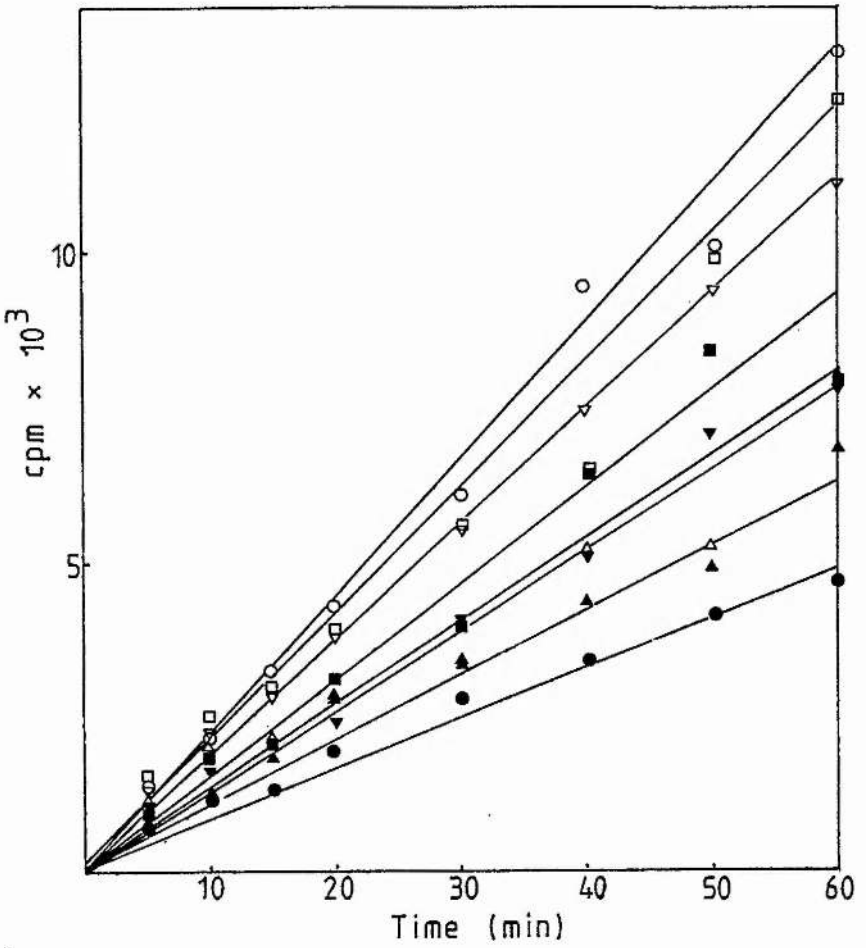

Fig. 67

Incorporation of uridine under bacteria-free labelling conditions in the presence of metabolic inhibitors.

- △ zero
- ▲ 0.2µg/ml actinomycin D
- ■ 0.5µg/ml actinomycin D
- □ 1 µg/ml actinomycin D
- ▼ 2 µg/ml actinomycin D
- ● 5 µg/ml actinomycin D
- ○ 1 µg/ml ∝- amanitin
- ▽ 5 µg/ml ∝- amanitin

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The possibility that cells do not take up actinomycin D in MS but only in bacterial medium by phagocytosis was ruled out by the following experiment. Paramecia were incubated in various concentrations of actinomycin D in bacterised medium for 1 hour before being washed twice with MS and resuspended as a 0.5% suspension in MS containing actinomycin D at the same concentration and tritiated uridine. In addition, two samples were similarly treated with two concentrations of α - amanitin which should inhibit mRNA production (Lindell et al., 1970). Again the inhibitors do not appear to have any effect on uridine incorporation (Fig. 67). Each sample shows linear incorporation at a different rate, some higher and some lower than the control (inhibitor-free) sample. The rate of incorporation is not dependant on the concentrations of the inhibitor and the variation is probably due to different numbers of cells in each sample.

Bacteria-free labelling of RNA with uridine, although avoiding the difficulties of interpretation of the results of bacterial labelling, clearly has its own problems of interpretation. The normal pattern of labelling observed in most organisms i.e. rapid labelling of heterogenously sized mRNA and ribosomal RNA precursor, becoming obscured by the labelling of more stable rRNA and tRNA, is not seen here. Rather there appears to be fairly consistent labelling of three size classes of RNA: 4S which probably corresponds mainly to tRNA, a peak at approximately 18S which may correspond to 18S rRNA (although it does not appear to be methylated) and a 20-22S fraction.

There is generally relatively little labelling in the region in which one would expect to find the ribosomal precursor (34S), and little labelling of 25S RNA, although this could be obscured by the 20S peak. There is also little labelling of mRNA which should be evident in the 4-18S region (see this Chapter, section i).

The 20S rapidly labelled RNA does not appear to be polyadenylated but is highly methylated. It is probably cytoplasmic in origin for two reasons. Firstly, it is not present in RNA preparations from labelled nuclei. Secondly, it does not appear consistently in RNA extracted from the post mitochondrial supernatant. For this latter reason and because its synthesis is inhibited neither by actinomycin D nor by ∝ amanitin it is probably mitochondrial in origin. (Attardi et al., 1970, Kuntzel and Schaffer, 1971). In Tetrahymena the large mitochondrial ribosomal RNA has a size of 21S (Chi and Suyama, 1970). The smaller mitochondrial ribosomal RNA species should sediment at 14S and, if the same size in Paramecium, is either synthesised in low amounts or obscured by labelling of 18S ribosomal RNA. If the 20S peak is indeed mitochondrial RNA, it is difficult to explain why it should be so predominant in such labelling experiments. Possibly the conditions used either stimulate mitochondrial DNA transcription or inhibit macronuclear transcriptional activity, possibly as a response to harvesting or to the labelling conditions, for instance the formate ion. Macronuclear RNA synthesis is not completely inhibited however since labelling paramecia for 2 hours followed

Fig. 68

Micrograph of a spread preparation of monoxenically cultured Paramecium showing two types of transcription unit. On the left are chromatin axes (CA) with beaded RNP 5-12nm in diameter and on the right chromatin axes with smooth (Sm) RNP, 20nm in diameter.

(Magnification 18,476X)

Fig. 69

Micrograph of a transcription complex with fewer chromatin axes. It is possible to see RNP fibrils attached to the chromatin axis. (shown by arrows).

(Magnification 13,410X)

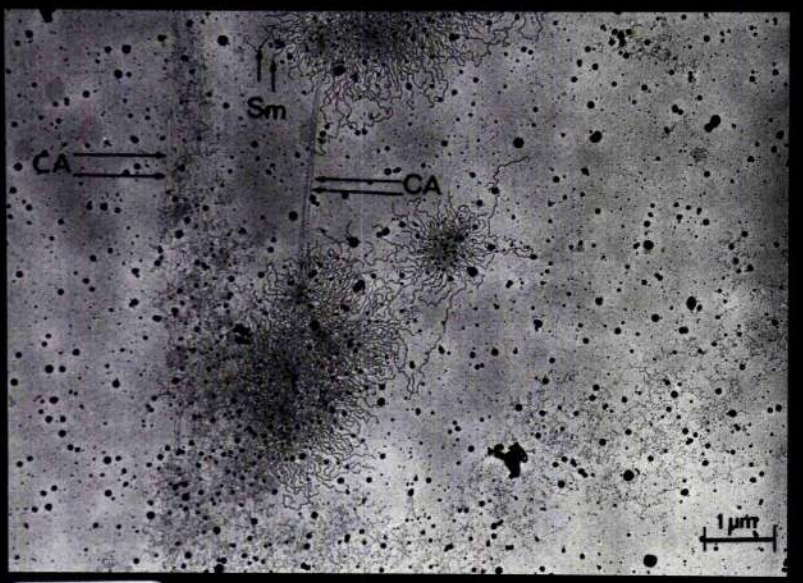

Fig. 68

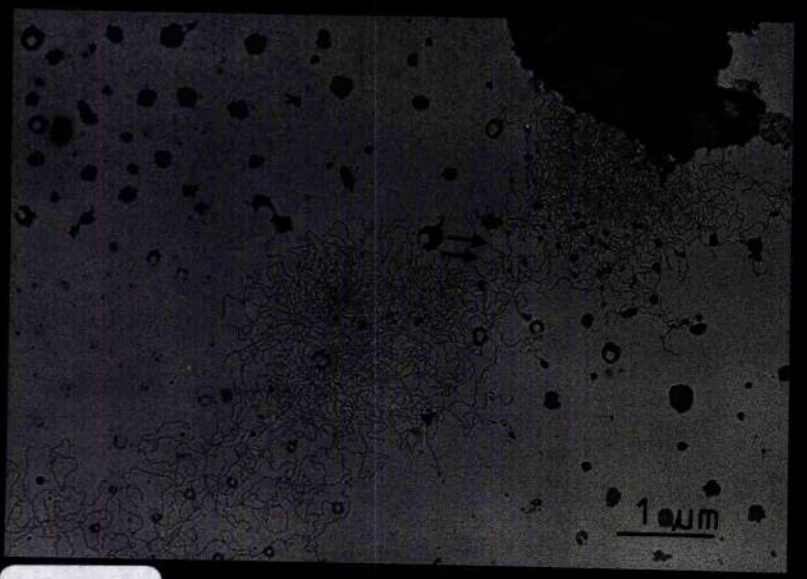

Fig. 69

by a 2 hour chase results in labelling of the 25 and 18S peaks. Secondly, labelled polyA$^+$RNA can be recovered from labelled cells. Successful labelling of ribosomal RNA and tRNA has been obtained under similar labelling conditions by Cummings (1975).

(c) Visualisation of RNA transcription

Cells were spread for visualisation of active transcription units by a modification of the method of Miller and Bakken (1972). Two types of transcription unit were observed (Fig.68). One type has beaded RNP fibrils of 5-12 nm diameter which appear similar to transcription units of other organisms (e.g. Hamkalo et al., 1973, McKnight et al., 1976). The other type of transcription unit has smooth 20nm diameter RNP fibrils, similar to those observed in Drosophila spermatocyte nuclei (Glätzer, 1980).

In both cases many chromatin axes lie in parallel making it difficult to assess the packing density of RNA polymerase molecules on each axis. In the case of the smooth RNP transcription units, RNP fibrils are densely packed and sometimes appear in isolation from the chromatin axis, possibly due to endogenous nuclease activity. Where there are fewer chromatin axes, it is possible to see the point of attachment of individual fibrils (Fig.69). In the particular transcription unit shown in Fig.69 the chromatin axis is densly packed with RNP fibrils and, presumably, with RNA polymerase molecules. The nascent transcript length is difficult to measure, but appears to be a few microns in length. This is larger than can be isolated as intact RNA (see section i, this Chapter) and may indicate

nicking of the primary transcript.

Transcription units of the two types were only observed in preparations of monoxenic cells. Axenic cells, which are metabolically less active, showed largely condensed chromatin. Unfortunately spread preparations of purified macronuclei showed no identifiable transcription units. It is probable that the method used to prepare macronuclei results in degradation of nascent transcripts.

Fig. 70

Fig. 70

Autoradiography of wheatgerm *in vitro* translation products analysed on a 15% SDS acrylamide slab gel.

In track 1 the sample had no added polyA$^+$ RNA (10 times the volume of sample used in track 2 was applied to track 1 to show endogenously synthesised polypeptides clearly.) In track 2 the sample had *Paramecium* polyA$^+$RNA added.

The molecular weights in kdaltons of the major *Paramecium* bands are shown.

(iv) Cell-free protein synthesis using Paramecium RNA

(a) Protein synthesis in a wheat-germ in vitro system

A sample of polyA$^+$RNA from G cells was translated in a cell-free synthesis system derived from wheatgerm (Roberts and Patterson, 1973). The RNA was found to stimulate the incorporation of ^{35}S-methionine into TCA precipitable material by a factor of 5-10 times greater than that of the incubate minus Paramecium polyA$^+$RNA. The products of the in vitro reaction were subjected to electrophoresis through a 15% polyacrylamide SDS gel. Incorporation into molecular size categories of protein was examined by autoradiography of the dried gel (Fig. 70). Incorporation in the absence of added Paramecium polyA$^+$RNA is shown in the adjacent track. This contains ten times the volume of incubate compared to the Paramecium sample in order to show endogenously - synthesised proteins clearly. The incubate containing Paramecium polyA$^+$RNA translates some proteins which are not present in the incubate lacking polyA$^+$RNA. The major bands have molecular weights of 16k, 17k, 22k, 29k, 30k and 34k daltons. In addition a faint band is seen at 46-48k daltons. There is considerable material synthesised which has a molecular weight of less than 15k daltons. This may be due to the translation of short polyA$^+$RNA since the size distribution of this sample of polyA$^+$RNA, which had been isolated from PMS, showed that much of this material was approximately 4S in size (see Fig. 27). Hruby et al. (1977), using a different extraction technique, later adopted in this

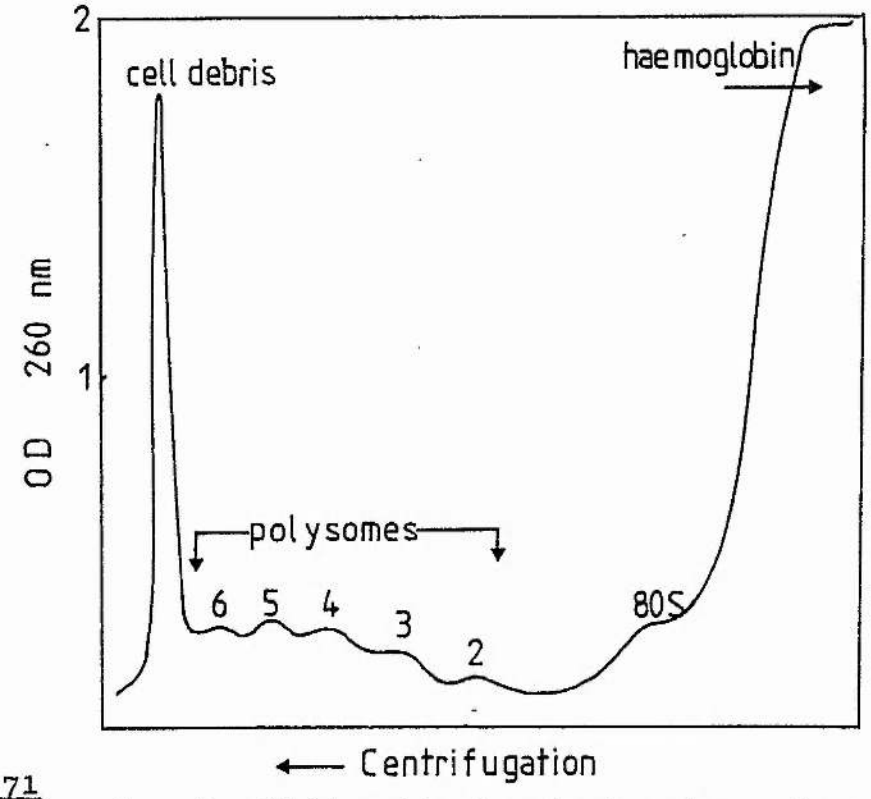

Fig. 71
A sample of rabbit reticulocyte lysate analysed by sucrose gradient centrifugation. Polysomes ranging from dimers to hexamers can be detected as peaks.

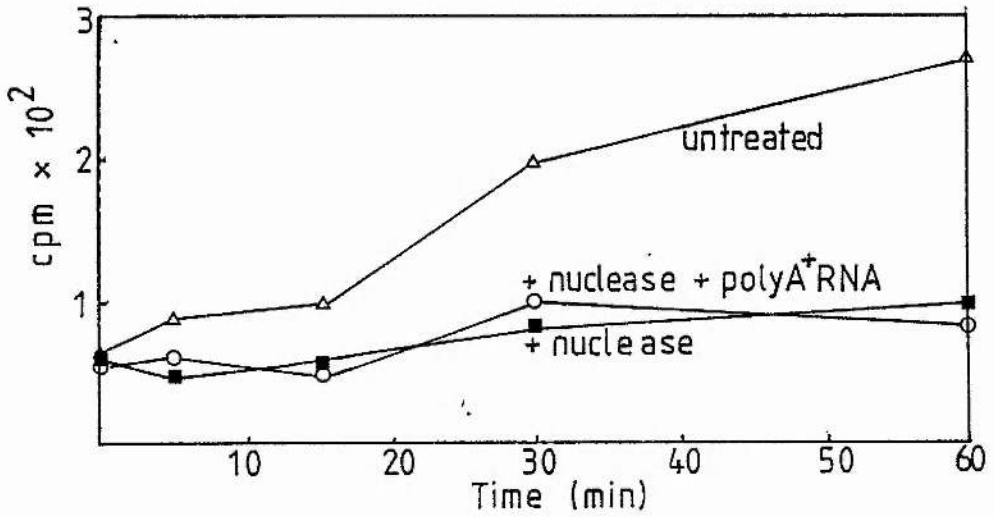

Fig. 72
The incorporation of ^{35}S-methionine by samples of reticulocyte lysate.
△ - untreated . ○ - treated with micrococcal nuclease
■ - treated with micrococcal nuclease and with the addition of oocyte polyA+RNA

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work, obtain polyA$^+$RNA of greater average size. This also translates successfully in a wheatgerm *in vitro* synthesis system and an autorad shows that proteins of size range 12-70 kdaltons are synthesised, the higher size range of labelled protein probably being a direct consequence of the higher size range of the polyA$^+$RNA.

(b) *Protein synthesis in a rabbit reticulocyte lysate*

The rabbit reticulocyte lysate was investigated since it appears to be more efficient in translating large mRNAs (Pelham and Jackson, 1976). If the *Paramecium* surface antigen is one molecule (see Chapter IV), then it will be coded by a large messenger RNA. The reticulocyte system also has the advantage of low levels of endogenous incorporation.

The reticulocyte lysate was prepared as described by Pelham and Jackson (1976). A sample of the lysate was examined on a polysome gradient prior to storage at -80°C. As seen from Fig. 71, the lysate contains polysomes ranging in size from dimers to hexamers, typical of globin mRNA containing polysomes.

The activity of the lysate was tested with polyA$^+$RNA isolated from *Triturus cristatis* oocytes. Three samples were analysed; the first consisting of a sample of lysate which was left untreated with micrococcal nuclease; the second treated with micrococcal nuclease but with no added polyA$^+$RNA; the third treated with micrococcal nuclease and supplemented with 50µg/ml oocyte polyA$^+$RNA. All three samples contained a ^{14}C-labelled mixture of amino acids. The samples were incubated at 37°C with aliquots taken at time intervals,

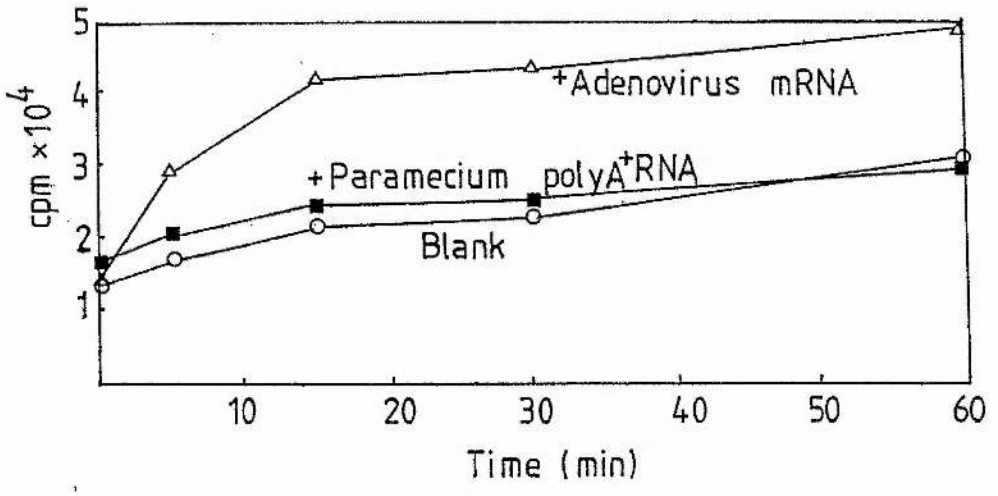


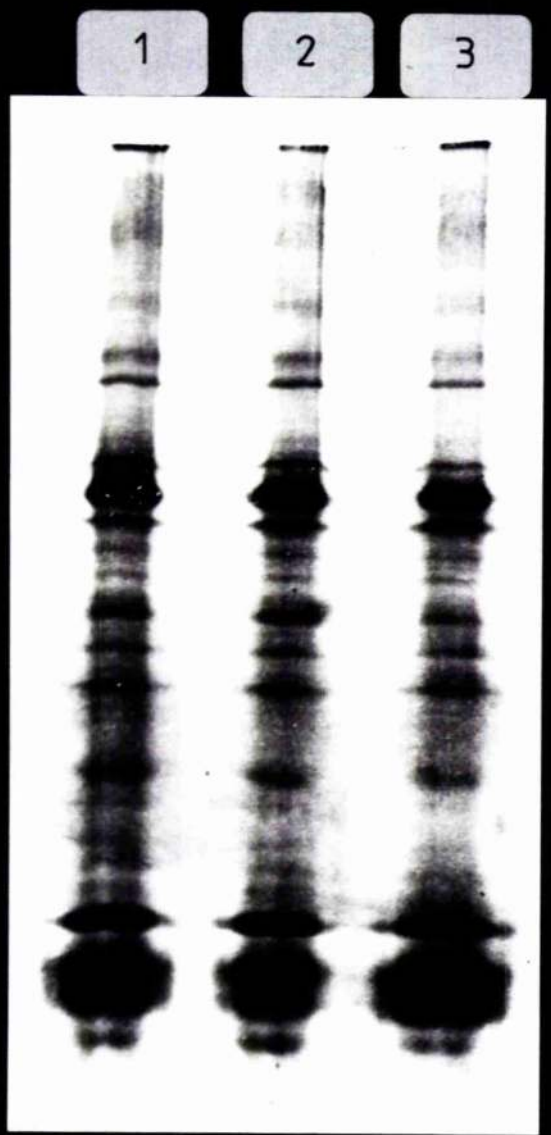
Fig. 73

The incorporation of ^{35}S-methionine by reticulocyte lysate obtained from New England Nuclear biochemicals

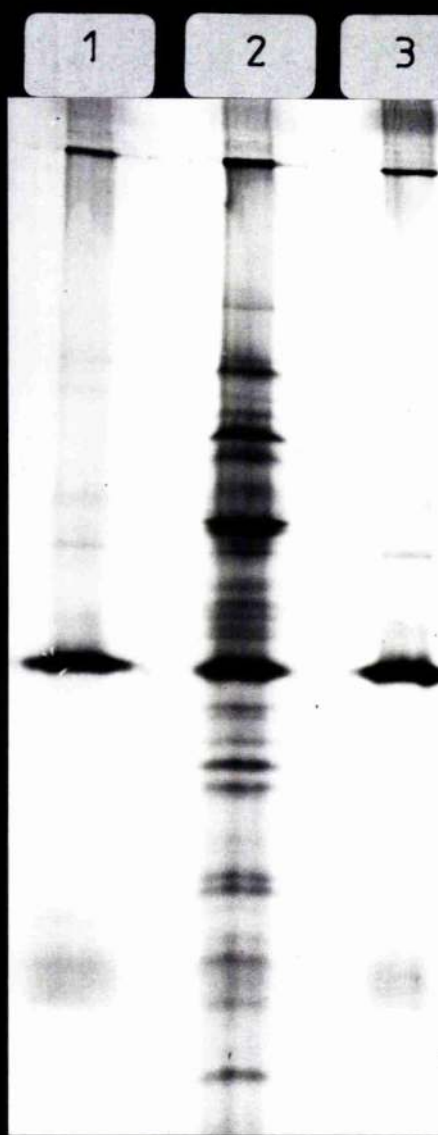
○ - no added RNA

△ - + adonovirus mRNA

■ - + Paramecium polyA$^+$RNA



Acrylamide gel



Autorad

Fig. 74

Fig. 74

Acrylamide gel and autorad of rabbit reticulocyte *in vitro* translation products analysed on a 12.5% SDS acrylamide slab gel.

Track 1 no added RNA
Track 2 + adenovirus mRNA
Track 3 + *Paramecium* polyA$^+$RNA

treated with NaOH to remove charged tRNAs and precipitated with TCA. As seen from Fig. 72, the untreated lysate incorporated labelled amino-acids into TCA precipitable material. Incorporation was markedly reduced by treatment with micrococcal nuclease, as expected. However, supplementing the treated lysate with polyA$^+$RNA did not increase incorporation above the endogenous level, suggesting that either the lysate has been rendered inactive by the treatment, or the polyA$^+$RNA is unable to stimulate incorporation. This result was found with a number of different lysate preparations using a variety of different polyA$^+$RNA preparations. Consequently a sample of commercial lysate was used (New England Nuclear Biochemicals). As a control adenovirus mRNA (supplied with the lysate) was added to the lysate. This stimulated incorporation as shown in Fig. 73. However the addition of polyA$^+$RNA from Paramecium did not stimulate incorporation. As seen from the autorad (Fig. 74), while the lysate containing adenovirus mRNA translates many proteins, only a few are seen in the lysate with added Paramecium polyA$^+$RNA and these are identical to those of the blank (no added polyA$^+$RNA). The prominent band in the three tracks probably corresponds to globin.

The addition of Paramecium polyA$^+$RNA to a second commercial lysate (Radiochemical Centre, Amersham) also caused no stimulation of radioactive incorporation. PolyA$^+$RNA was prepared very carefully for addition to the reticulocyte lysate by the stepwise treatment with deoxyribonuclease, proteinase K and the removal of SDS and residual proteins by

pelleting through CsCl. RNA prepared in this way had a good size profile, the majority having S-values greater than 10S. Nevertheless this RNA failed to stimulate incorporation in the reticulocyte system. This is in contrast to the situation with the wheatgerm system. Here the polyA$^+$RNA used was the bound fraction from an oligo(dT)-cellulose column, was not further purified and was mostly smaller than 10S in size. Yet this polyA$^+$RNA translated successfully in the wheatgerm system.

While it is not possible to rule out total inactivity of the reticulocyte lysates used, nor the possible sensitivity to some inhibitory factor in the purified polyA$^+$RNA, (possibly traces of cesium ion), the evidence suggests that polyA$^+$RNA from Paramecia cannot be translated by the reticulocyte system. While the wheatgerm system has been shown to translate mRNA from a wide variety of sources, reticulocyte lysate has generally been used for the translation of mRNA from viruses and animals. There is less data available on the in vitro translation of mRNA from plants or fungi. It is possible that some factor is missing in the reticulocyte system which would enable it to translate ciliate mRNA.

4. DISCUSSION

The RNA of *Paramecium* has many eucaryotic features, but in some respects differs from that of the higher eucaryotes. For example, although *Paramecium* ribosomes approach 80S in size, the ribosomal RNA molecules are smaller than those of higher eucaryotes and the protein: RNA ratio is less, being intermediate in size between that of bacteria and higher eucaryotes (Reisner *et al.*, 1968).

A small percentage of *Paramecium* RNA is polyadenylated, clearly a eucaryotic feature. This polyA$^+$RNA has a size range similar to that found in higher eucaryotes. The size distribution of *Paramecium* polyA$^+$RNA is similar to that of rapidly labelled RNA from *Tetrahymena* (Prescott *et al.*, 1971). If the species of ciliate are comparable in this respect, this suggests that polyA$^+$RNA is not transcribed as a high molecular weight precursor. In order to investigate whether this is true for *Paramecium*, RNA synthesis has been examined both by radioactive labelling and by visualisation of actively transcribing chromatin.

There are clearly problems to be expected in the labelling of cells which feed on bacteria. Bacteria will incorporate most labelled metabolites and, while this is useful as a labelling technique in itself, complicates the interpretation of the labelling of paramecia in the presence of bacteria. One cannot, with certainty, attribute a particular pattern of labelling to that of the paramecia rather than to the bacteria.

Two labelling methods have been used to overcome this problem although neither is without its disadvantages. Initially paramecia were labelled with labelled bacteria and

the post-mitochondrial supernatant prepared. This should exclude any intact bacteria but has the disadvantage that it is not an ideal method for RNA extraction, nor can the possibility of bacterial RNA breakdown products be excluded. Such material would have a high specific activity and only a small amount of contamination could obscure the labelling of Paramecium RNA.

However, in spite of these difficulties, it appears that much of the labelled RNA appearing in the cytoplasm is initially smaller than 18S and that cytoplasmic ribosomal RNA is labelled to a noticable extent only after 1 hour of labelling. In general terms this labelling pattern is similar to that of most cells.

The alternative method of labelling was that used by Cummings (1975) to label ribosomal and tRNA. In this method cells are incubated in a bacteria-free non-nutrient medium in the presence of ^{3}H-uridine. Here, the levels of incorporation are lower but, since bacteria are absent, total cellular RNA can be extracted using Kirbys buffer, which appears to preserve better the extracted RNA. However, this RNA, in contrast to that from the previous method, contains nuclear, mitochondrial as well as cytoplasmic RNA.

By this second method, the majority of labelled RNA synthesised at 25^{o}C sediments at 25-18S. 18S RNA appears to be more highly labelled than 25S RNA, even after 4 hours, although stable RNA smaller than 18S may be contributing to this effect. The labelling of the 25S peak increases with time, although it appears to be obscured by the labelling of an RNA species in the size range 20-22S. The 4/5S peak is

very effectively labelled by this method but there is relatively little labelling of material between 4S and 18S. This is surprising since the majority of polyA$^+$RNA would be expected in this region and, in Tetrahymena this region was highly labelled after a 5 minute pulse (Prescott et al., 1971).

This labelling pattern is consistently found in cells labelled at 25°C, even with short labelling times. At 32°C a similar pattern is found although here, rather than the three peaks of labelling at 20-22S, 18S and 4/5S, two peaks at 16-20S and 4/5S are seen for labelling times ranging from 5 minutes to 1 hour. PolyA$^+$RNA isolated from cells labelled for 1 hour at 32°C shows a heterogenous pattern of labelling with a peak at 12S which corresponds to the optical peak of the polyA$^+$RNA material.

This pattern of labelling of RNA obtained with this method is unlike that found in other eucaryotic cells or indeed like that of Tetrahymena (Prescott et al., 1971). Of particular interest is the prominent 20S peak. A number of features point to this being mitochondrial ribosomal RNA. Firstly, it is not observed consistently in RNA from the PMS, suggesting that it is either nuclear or mitochondrial in origin. It is however not found in labelled nuclear RNA. It is not polyadenylated but is highly methylated. Mitochondrial ribosomal RNA in Tetrahymena sediments at 21S and 14S (Chi and Suyama, 1970) and so the 20-22S species found in Paramecium most probably corresponds to the large mitochondrial ribosomal RNA.

There is no clear indication of a labelled 14S species, but this could be contributing to the labelling of the 18S peak, which may also include ribosomal RNA transcribed in the macronucleus.

Whether this pattern of labelling is due to a stimulating effect of this method of labelling on mitochondrial RNA synthesis or to an inhibitory effect on nuclear RNA synthesis is not known. It is however unlikely, if the effect is inhibitory, that such inhibition is total, for a number of reasons. Firstly, although using Dryls solution rather than MS, Cummings (1975) successfully labelled ribosomal and tRNA to high specific activities in similar conditions. Secondly, polyA$^+$RNA isolated from labelled cells is labelled although to a fairly low level. Thirdly, cells labelled for two hours followed by a 2 hour chase in cold bacterised medium show a labelling profile coincident with the optical profile, suggesting that 25 and 18S RNA are synthesised and that they are more stable than the 20S species.

There are considerable problems, therefore, with the interpretation of the labelling data and more work would be required to ascertain exactly what is happening in the bacteria-free labelling conditions. One feature of interest, regardless of the labelling method, is the absence of labelled material larger than 30 S, except for some labelling of the 34S region. This suggests that, as with Tetrahymena, there is no rapidly labelled material larger than the final polyA$^+$RNA. As in Tetrahymena the 34S peak is probably ribosomal RNA precursor. In the nucleus, although there appears to be some breakdown during the preparation, labelled material is also smaller than 30S.

The absence of a rapidly-labelled high molecular weight nuclear RNA (hnRNA) is in accord with the observations in other lower eucaryotes. In Amoeba (Prescott et al., 1971) and Physarum (Braun et al., 1966) no high molecular weight precursors are found. In Dictyostelium nuclear RNA is only slightly larger (by 20%) than cytoplasmic RNA (Firtel and Lodish, 1973). Lower eucaryotes may have a simpler mechanism of processing primary transcripts. The smaller size and rapid processing of the ribosomal RNA precursor in Tetrahymena tends to support this possibility.

RNA synthesis in Paramecium has also been examined by visualisation of transcription units according to the method of Miller and Bakken, (1972). Two types of transcription unit are observed, one with normal 5-10nm beaded RNP fibrils and the other with smooth 20nm RNP fibrils. Transcription units of the latter type have been observed in the nuclei of spermatocytes of Drosphila hydei (Glätzer, 1980). Since the two types of transcription unit occur in close proximity, the differences are unlikely to be due to local spreading conditions. In both cases, several chromatin axes lie adjacent and make it difficult to determine the fibril density, the maximum RNP fibril length or transcription unit length. Nevertheless, the packing density of RNA polymerases, as estimated from regions where individual RNP fibrils are distinguishable, appears to be high. Some of the longer smooth RNP fibrils would appear to have lengths of up to 2-3μm. If the RNA/RNP packing ratio is similar to that of the smooth fibrils found in Drosphila hydei (8.4) then this would indicate that

the primary transcript is much longer than any RNA which can be extracted from the cell. This is a general observation (e.g. Sommerville, 1980) and indicates nicking or very rapid processing of the RNA while it remains attached to the chromatin axis. It is interesting that Paramecium which appears to lack a defined hnRNA precursor should, like other organisms, have long nascent transcripts.

Another aspect of RNA synthesis which has been investigated is the sequences of polyA$^+$RNA which are present in cells grown at 25°C and 32°C. The macronuclear genome is sufficient to code for up to 8×10^4 "genes". Saturation experiments suggest that only 5% of the genome is transcribed into polyA$^+$RNA in cells grown at 25°C and a higher value of 7.6% in cells grown at 32°C. The reason for the higher value is not apparent, but does appear to be a real phenomenion since the data on cDNA-polyA$^+$RNA hybridisation also suggest that a larger percentage of the genome is transcribed in cells grown at 32°C. This may be some effect of the increased metabolic rate and instability of certain proteins at higher temperature.

PolyA$^+$RNA - homologous cDNA hybridisation of sequences from both G and D type paramecia, indicate that different polyA$^+$RNA sequences are present at different intracellular concentrations. For the purposes of analysis, the polyA$^+$RNA of Paramecium is fitted into three frequency classes, although this is a somewhat artificial division and it is possible that polyA$^+$RNA frequencies cover a broader and more continuous range. The least frequent class of polyA$^+$RNA sequences

accounts for most of the sequence complexity of the RNA. In D cells, the fraction of the RNA in this class is slightly lower than in G cells (47% as opposed to 56%) but appears to have a higher complexity.

Complexity values of all three classes of RNA are subject to errors from a variety of sources (discussed by Bishop et al., 1974., Birnie et al., 1974). It is assumed that the final level of cDNA hybridisation, in this case 75% is the maximum level. RNA sequences hybridising very slowly might increase the level of hybridisation only slightly but could contribute significantly to the complexity of the least frequent class of polyA$^+$RNA. Any error in the value of the Rot$\frac{1}{2}$ of the cDNA polyA$^+$RNA hybridisation of reticulocyte polyA$^+$RNA, which has been used as a standard, will naturally result in errors in the value of the complexity of each frequency class of polyA$^+$RNA. In addition, contaminating polyA$^-$RNA in the polyA$^+$RNA sample will not contribute to the hybridisation reaction but would increase the apparent Rot value of each transition. It is further assumed in the calculations that the GC content, poly(A) content and the average length of both the polyA$^+$RNA and the cDNA copy are similar for each frequency class of RNA. This may not be the case, particularly in the least frequent class of RNA since the correction factors are based on the properties of the more frequent RNA species. Such errors, cumulatively, could lead to considerable variation in the values, particularly

in the least frequent class of RNA and so the values should be regarded as approximate .

If one calculates the number of molecules of each different RNA species, assuming the mean size to be 2000 nucleotides, then there appears to be 1-4 x 10^3 copies of each of the least frequent RNA species per cell. This is a very high number in comparison with that of other cells where generally the least frequent RNA species are present only 1-10 times per cell (e.g. Axel et al., 1976, Getz et al., 1976). The higher value in Paramecium may be due to the polyploid nature of the macronucleus which contains 840 copies of each "unique" gene. If each of these is being transcribed and the half-life of each transcript is at least as long as the time taken to transcribe each gene, then one would expect there to be in excess of 1000 copies of each RNA species per cell at any one time. Very little is known about the stability of RNA species in Paramecium, although it has been reported that the antigen mRNA may be stable for up to 16 hours (Sommerville, 1969). The rate of transcription, as judged by examination of transcription complexes , also appears to be high and so it is not unreasonable that some species of RNA, for example the more frequent RNA species, could be present in hundreds of thousands of copies per cell.

The saturation data suggest that some sequences present in G cells are not present in D cells and vice versa. This is confirmed by an examination of heterologous cDNA- polyA$^+$RNA hybridisation. RNA sequences which are present in one cell

type but absent in another, cover a broad range of frequencies. This is similar to the situation in other cell types where, again, the differences in the RNA sequences present cover a broad range of frequencies (e.g. Ryffel and McCarthy, 1975., Axel et al., 1976).

In this study the complexity of polyA$^+$ RNA has been studied. No data are available on polyA$^-$RNA, although this would be an interesting topic to pursue. The work of Gibson (1970) who hybridised rapidly labelled RNA with DNA suggests that the total percentage of the genome transcribed into rapidly labelled RNA is not much greater than the percentage of the genome transcribed into polyA$^+$RNA. This appears also to be the case in Tetrahymena (Christianson, 1970).

PolyA$^+$RNA from cells grown at 25°C could be successfully translated in a wheatgerm in vitro protein translation system (Roberts and Patterson, 1973) although apparantly not in the rabbit reticulocyte cell-free system (Pelham and Jackson, 1976) which may lack some factors essential for translation of ciliate sequences. The proteins translated in the wheat-germ system have a broad range of size, up to 45 kdaltons being found. Most of the translated proteins are small in size but this is probably due to the correspondingly small polyA$^+$RNA used in the translation system.

Although much work remains to be carried out to elucidate the details of RNA synthesis in Paramecium, the data in this Chapter provides a basis for the examination of changes in RNA synthesis which may occur during serotype transformation. The complexity data in particular suggests that there are considerable differences in the spectrum of RNA present in

cells grown at 25°C and 32°C. In Chapter IV changes in RNA and protein synthesis which occur during transformation of G cells to D cells are investigated.

5. SUMMARY

RNA was extracted from Paramecium both from intact cells and from a polysome-containing fraction and the RNA analysed by sucrose gradient centrifugation. PolyA$^+$RNA was isolated from total RNA and the different sequences of RNA present analysed by cDNA-polyA$^+$RNA hybridisation. The polyA$^+$RNA of Paramecium falls into a broad range of intracellular frequencies which can be divided into three frequency classes. The least frequent class is transcribed from 10-20% of the genome and each different sequence is present approximately 1000 times in each cell. The higher frequency classes of polyA$^+$RNA are transcribed from only 0.1-0.2% of the genome but each sequence is present 10^5-10^6 times per cell.

PolyA$^+$RNA was further analysed by translation in two cell-free protein synthesis systems. Only in the wheat-germ system was protein synthesis stimulated and polypeptides up to 45 kdaltons in size were translated.

The synthesis of RNA in Paramecium was examined using two methods of radioactive labelling; using labelled bacteria and under bacteria-free conditions. Although neither method was completely satisfactory, the labelling pattern suggest that no high molecular weight precursor to mRNA in present. However, an examination of transcription units suggests that the nascent RNP fibrils on Paramecium chromatin are considerably longer than the molecular lengths of isolated RNA.

CHAPTER IV

Molecular Events during Serotype Transformation

1. INTRODUCTION

The period during which control of the antigen genes can best be investigated is the period of serotype transformation. During this time there is a switch from the expression of one gene to that of another.

A number of factors can cause or influence transformation of one serotype to another. These include temperature, salt concentration, pH, stage in the life cycle, type of culture medium, quantity of food, treatment with homologous antiserum, normal serum, proteolytic enzymes, UV- and X- irradiation, the chemicals patulin and acetamide and the metabolic inhibitors actinomycin-D, chloramphenical and puromycin (Reviewed by Finger, 1964).

One of the best studied factors in determining the activity of antigen genes, particularly in P. primaurelia is temperature. In P. primaurelia there is a simple relationship between the temperature of a culture and the serotype expressed. In general terms, the S serotype is found at temperatures below $18^{o}C$, the G serotype at temperatures between $18^{o}C$ and $27^{o}C$ and the D serotype above $27^{o}C$ although different alleles have different and characteristic ranges of stability (Beale, 1954).

A culture expressing one serotype can be induced to transform to the expression of another serotype simply by changing the temperature of the culture medium. The kinetics of the transformation process depend on the nature of the environmental change, a rapid or large change in temperature causing the culture to transform more rapidly than when a slow or small change is made

(Beale, 1957). In some stocks there is a time-lag of several fissions before the onset of the change in serotype, but in stock 168 there is little, if any, delay in the onset of transformation after the temperature change.

In several stocks of Paramecium the new serotype may be detected, by immobilisation of the cells with antiserum, within 12 hours of the transformation stimulus (Mott, 1965). However, immobilisation is a relatively insensitive method for detecting antigen synthesis since it requires that sufficient antigen be synthesised to cover a large part of the cells surface before it can be detected. The most sensitive assay method is that of autoradiography of immunoelectrophoresis gels (Sommerville, 1968). By this method the new antigen can be detected within 15 minutes of the temperature change (Sommerville, 1969).

In an attempt to elucidate molecular events during transformation, the effect of a number of metabolic inhibitors have been investigated. Actinomycin D, used at a concentration sufficient to inhibit growth and division, also inhibited transformation (Austin et al., 1967). A similar result was obtained with chloramphenicol, indicating that both RNA and protein synthesis are prerequisites of transformation. In the experiments of Austin et al. (1967), transformation was assayed by immobilisation. Using the more sensitive assay technique of autoradiography of immunoelectrophoresis gels, Sommerville (1969) showed, in contrast, that the new antigen was synthesised after transformation in the presence of actinomycin D, suggesting the presence of pre-existing mRNA. However, it is possible that actinomycin D,

in the conditions used, is not fully inhibiting RNA synthesis and that enough mRNA is being synthesised to account for the small amount of antigen which can be detected, but not sufficient to allow for the translation of the amounts of antigen which would be required to be detected by immobilisation in the experiments of Austin et al. Austin et al. (1967) in fact note that 12.5 μg/ml actinomycin D inhibits RNA synthesis only to the extent of 60%.

The antigen molecule itself is an extremely large protein, 310,000 daltons in size. There is some disagreement about whether or not it is composed of subunits. Steers (1965), from biochemical evidence, considers the antigen to be composed of three identical subunits, each composed of three different subunits. The smallest subunit has a size of 35,000 daltons Finger et al. (1966), from observations on the immunological behaviour of heterozygous clones, also suggested that the antigen must be composed of subunits. However, Reisner et al. (1969) consider that the antigen is a single polypeptide. Hansma (1975) agrees with this finding, suggesting that the reason earlier workers find subunits is due to the purification, with the antigen, of a β-mercaptoethanol stimulated protease.

If the antigen is indeed a single polypeptide, then the mRNA coding for the antigen would have a minimum size of 2.8×10^6 daltons. This would have a sedimentation value of approximately 40S. Were the antigen made up of three subunits, the mRNA would sediment at approximately 21S and if composed of nine subunits, the predicted sedimentation value would be approximately 12S.

Using a cell free synthesis system capable of translating Paramecium polysomes, Sommerville (1970) has shown that the

fraction most active in antigen synthesis is the membrane-bound polysome fraction. In addition there is some antigen synthesis by large polyribosomes which sediment at 200-300S. The treatment of PMS with deoxycholate to release membrane-bound polysomes enhanced the synthesis of antigen by the large polyribosome region, suggesting that antigen is normally synthesised by large polyribosomes, the majority of which may be bound to membranes.

By probing a polysome gradient with antibodies to the antigen protein, which had been labelled with iodine ^{125}I, Sinden (1973) showed that the antigen is specifically associated with polysomes o. sizes 228S and 311S, which would contain 10-11 and 34-35 monosomes respectively. Such polysomes would be expected to translate proteins of size 35,000 daltons and 110,000 daltons respectively which are the sizes of 1 and 3 antigen subunits respectively. Sinden suggests that the 311S polysomes arise from the aggregation of three 228S polysomes by virtue of the nascent polypeptides and that the intact antigen 310,000 daltons may be formed by disulphide bonding at the site of synthesis. The observation that some heterozygous clones form only one hybrid antigen (Finger et al., 1966) again suggests that the i-antigen subunits making up the final complete molecule are synthesised in close proximity. The association of newly synthesised polypeptides with pre-existing subunits is also suggested by the work of Sommerville (1967) who found that, although partially incomplete, polypeptides synthesised in vitro had similar immunological, electrophoretic and sedimentation properties to that of complete

antigen. These observations suggest that the complete antigen may form spontaneously without any special controlling influences.

The previous chapters have shown that the macronuclear genome contains sufficient DNA to code for 8 x 10^7 "genes", but that only 10-20% of this DNA is transcribed. The nucleotide sequence complexities of the RNA populations from cells expressing the G and D serotypes indicate a considerable difference in gene expression between the two serotypes and suggest that transformation may be more complex than a simple gene switch.

A major change in gene expression may partly explain the concept of the "cytoplasmic state" (Beale, 1957., Sonneborn, 1960). The cytoplasmic state is affected by a number of factors which include the genetic background, the recent history of the cytoplasm and various environmental conditions. The cytoplasmic state in turn influences the expression of the antigen genes: transformation appearing to be initiated by a change of cytoplasmic state, each state favouring the expression of a particular antigen gene. A change in cytoplasmic state may be associated with major changes in gene expression.

In this chapter, some of the molecular events occuring during transcription are investigated, particularly with regard to RNA synthesis, and an attempt is made to isolate and characterise the antigen mRNA molecule.

2. Materials and Methods

(i) Serotype Transformation

(a) Transformation of paramecia cultured at 25°C

To a culture of cells grown at 25°C and in late log phase

was added an equal volume of sterile grass medium heated to 42°-44°C. This increased the temperature of the culture to 32°C. The transformed culture was immediately placed in a waterbath at 32°C and later transferred to an incubator at 32°C. The serotype of the culture was determined at intervals, up to three days after transformation, by testing for immobilisation with both anti-G and anti-D sera as described in ChapterI, Materials and Methods. Additional sterile grass medium at 32°C was added daily to ensure continued growth and division of cells.

b) Transformation in the presence of inhibitors

Inhibitors were added to the 25°C culture prior to the addition of medium at 42°C. The final concentration of inhibitor ranged from 5-20 ug/ml. Actinomycin D (Calbiochem), 3-deoxyadenosine (cordycepin, Sigma) and α - amanitin (Boehringer) were used. In situations where the inhibitor markedly inhibited growth and division, additional medium was not added. Otherwise, the conditions of transformation were as described previously.

(ii) Labelling of cells with tritiated uridine and ^{14}C-amino acid mixture.

Each 0.1ml of packed cells, previously grown at 25°C, was washed and labelled in 100ml MS containing 10mM sodium formate with either 10µCi ^{3}H-uridine (27Ci/mmole) or 10µCi ^{14}C-amino acid mixture (57 Ci/mmole). Incubation was continued for 30 minutes at 25°C and 1ml samples removed at 5 minute intervals. The cultures were then divided into two and to one half 50ml of MS (containing 10mM sodium formate and either 5µCi ^{3}H uridine or 5µCi ^{14}C-amino acid mixture) at 42°C was added. Such cultures

were then incubated at 32°C. To the second 50ml of culture was added 50ml of MS (containing 10mM sodium formate and either 5µCi ^{3}H-uridine or 5µCi ^{14}C-amino acid mixture) at 25°C. These cultures were left at 25°C. Sampling was continued from all four cultures at 5 minute intervals until one hour after transformation and subsequently at 15 minute intervals for a further two hours. Samples from the ^{3}H-uridine labelled cultures were added to 1ml distilled water containing 0.1% SDS, 2ml of 10% TCA was added, and the samples placed on ice. ^{14}C-amino acid labelled samples were incubated at 37°C with 2ml of N NaOH containing 0.1% SDS. After 10 minutes 3ml of 10% TCA was added and the samples placed on ice. Samples were later filtered through GFA filters (Whatman), washed with 5% TCA and 96% alcohol, dried and counted in toluene based scintillation cocktail (NE 233, Nuclear Enterprises).

(iii) Short-term labelling of RNA with tritiated uridine during transformation

0.5ml of packed cells grown at 25°C were resuspended in 1ml MS at 32°C containing 10mM sodium formate, and incubated for 5 minutes at 32°C. 0.2µCi of ^{3}H-uridine (27 Ci/mmole) was added and the incubation continued at 32°C for 10 minutes. Cold uridine was added to a concentration of 15µg/ml, the cells harvested and added to a mixture of 7 parts Kirbys buffer, 2 parts sodium deoxycholate pH 9.5 and 1 part glycine buffer, pH 9.5. (For Kirbys buffer, see Chapter III). Cells were homogenised and extracted as described in Chapter III. A further 2 litre of culture at 25°C was transformed by the addition of 2 litre of sterile grass medium at 44°C. Two of the resulting

four litres were harvested 90 minutes after transformation, the cells washed and labelled with ^{3}H-uridine for 10 minutes and the RNA extracted as described previously. The remaining two litres of transformed culture was similarly treated 14 hours after transformation.

The RNA from each sample was recovered by centrifugation and analysed on a 15-30% NETS gradient. Fractions were recovered, precipitated with TCA, filtered through GFA filters and counted.

(iv) Gel electrophoresis of proteins from transformed cells

One litre of cells at 25°C was transformed as described previously. Samples of 500ml were removed from the transformed culture at 30 minutes, 3 hours, 8 hours and 24 hours after transformation. The cells were harvested, washed, resuspended in 10ml MS and packed tightly by recentrifugation at 5000rpm for 2 minutes in a conical tube. The supernatant was removed and 2ml 10% TCA added to the pellet. The precipitated cells were held on ice for 30 minutes, centrifuged and the precipitate resuspended in 2ml ice-cold 100% alcohol for 20 minutes with occasional mixing. The precipitate was recentrifuged for 2 minutes at 2,000rpm and resuspended in 2ml chloroform:methanol mixed in a 1:1 ratio. This was mixed on ice for 20 minutes and the precipitate recovered by centrifugation at 5,000rpm for 5 minutes. The pellet was dried and stored frozen until all samples had been taken. In addition 500ml of G culture and D culture were similarly treated, as was a sample of Klebsiella aerogenes which had been grown overnight in 100ml of limiting medium (see Chapter III).

Samples were dissolved in 100µl of 2% SDS, 0.1M dithiothreitol, 0.0625M Tris HCl, heated to 100°C for 5 minutes and 25µl applied

to a 15% acrylamide gel (see Chapter III). Electrophoresis was performed at 100V overnight and the gel was later stained, destained and dried down.

(v) Precipitation of polysome gradient fractions with antiserum

Cells were harvested, resuspended as a 0.5% suspension in 100ml MS and incubated at 25°C for one hour, 50mg of ^{35}S-labelled bacteria was added and incubation continued for two hours. Cells were washed free of labelled bacteria and incubated for one hour in 100ml medium containing unlabelled bacteria. The post-mitochondrial supernatant was prepared as described previously and 2ml of this was layered on a 15-30% polysome gradient with a 4ml 60% sucrose pad (see Chapter III). The gradient was centrifuged at 23,000rpm for two hours at 4°C and the gradient fractionated into 2ml fractions. Each fraction was dialysed against 0.9% NaCl and divided into two samples. To one set of samples was added 100µl anti-G serum and to the other 100µl of non-immune rabbit serum. Samples were incubated at 37°C for one hour, then 50µl of anti-rabbit goat serum (Welcome) added. Incubation was continued at 37°C for one hour. The immuno-precipitates in each sample were recovered by centrifugation at 15,000rpm for 10 minutes at 4°C rinsed twice with 0.9% saline, precipitated with TCA, filtered, dried and counted as described previously.

(vi) Isolation of RNA from precipitated polysomes

(a) Isolation of IgG from sera

Samples of about 1ml serum were extensively dialysed against 0.015M PB pH 8.0. The serum was then applied to a 30cm x 0.5cm

diameter column of DE52 Sephadex (Whatman) equilibrated in the same buffer. The eluate was passed through a flow cell monitoring at 280nm and the profile recorded on a chart recorder. IgG elutes in the void volume and was seen as a peak on the chart recording. The peak fraction was collected as approximately 5ml of solution and the IgG content was measured in a Pye-Unicam spectrophotometer monitoring at 280nm using the conversion 1mg/ml IgG equal to 1.40D. The serum was dialysed against polysome homogenisation buffer overnight. Anti-G and anti-D sera was also purified in this way. Anti-rabbit IgG (10mg/ml) which was used for secondary precipitation was obtained from Welcome Laboratories.

(b) Precipitation of polysomes and extraction of RNA

1ml of G-type cells was resuspended in 200ml MS containing 10mM sodium formate and 0.5mCi ^{3}H-uridine (27Ci/mmole) and incubated for 30 minutes. The cells were then harvested and the PMS prepared. The purified anti-G IgG was added and the mixture incubated for 1hr at 0°C. An equal amount of anti-rabbit IgG was added and incubation continued for a further one hour at 0°C. The immuno- precipitate was recovered by centrifugation at 4,000rpm for 10 minutes, the pellet raised in Kirbys buffer (with deoxycholate and glycine buffer as described previously) and phenol extracted. The supernatant was mixed with an equal volume of Kirbys buffer with deoxycholate and glycine buffer and also phenol extracted.

1.0ml of cells grown at 32°C was harvested and similarly treated.

The RNA samples from the pellets were raised in 100µl of NETS buffer, layered on a 15-30% NETS gradient and centrifuged

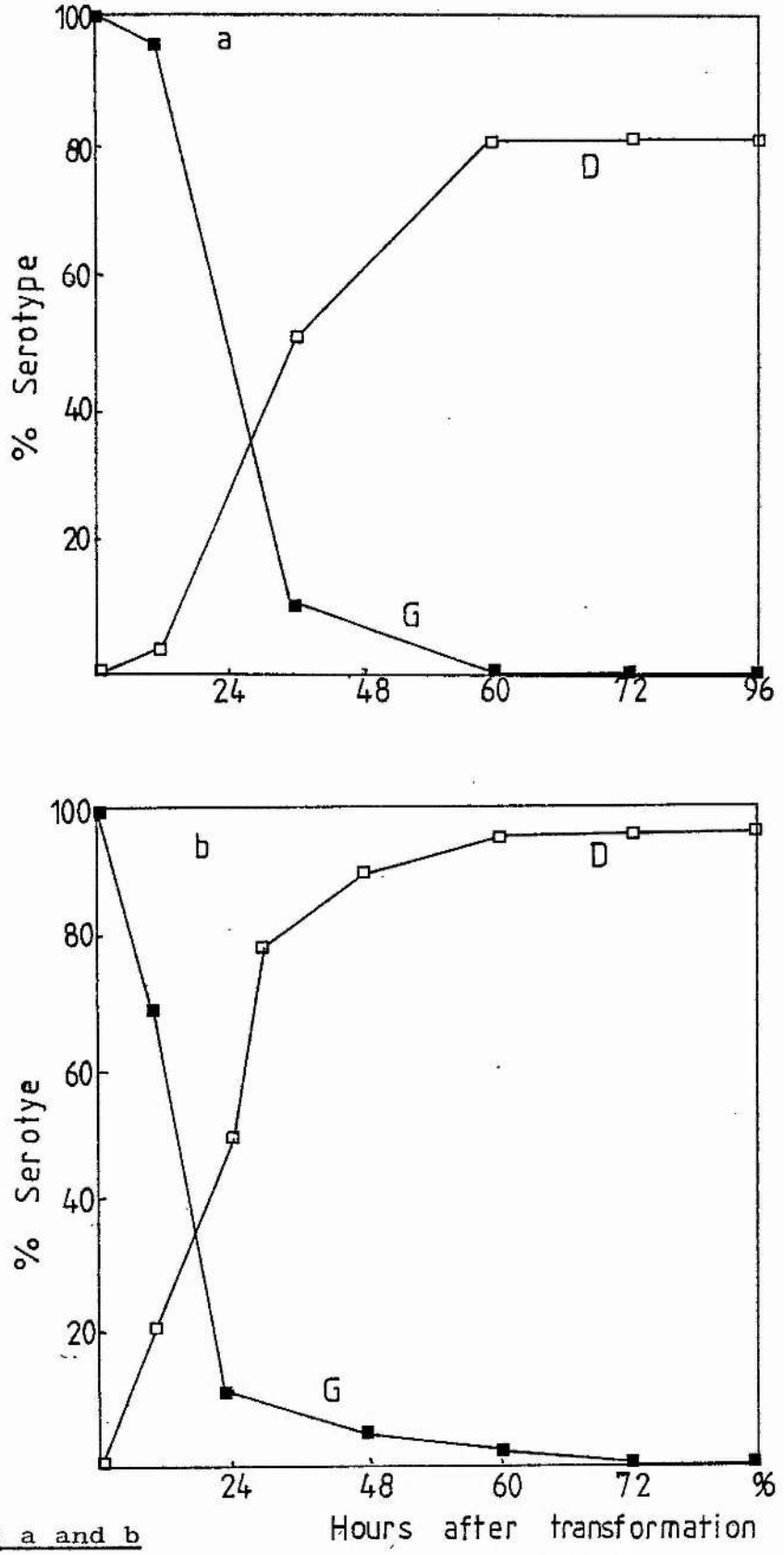


Fig. 75 a and b

The kinetics of transformation of a) 100ml culture and b) 1 litre culture.

■ % of cells with G serotype
□ % of cells with D serotype

at 22,000 rpm for 20 hours at 20°C. The gradient was fractionated and the fractions were TCA precipitated, filtered, dried and counted. Supernatant RNA was taken up in 0.5ml NETS buffer and 100µl layered on a gradient as above.

In a later experiment, the labelling and precipitation conditions were as described above, but RNA samples were raised in 0.1M Tris HCl, 10mM EDTA, 0.2% SDS, heated at 70°C for 5 minutes and applied to 15-30% sucrose gradients made up in the same buffer. Centrifugation and fractionation conditions were as described previously.

3. Results

(i) The kinetics of serotype transformation

Cells of stock 168 grown at 25°C express the G serotype. They can be transformed to the D serotype by rapidly increasing the temperature of the culture to 32°C and by subsequently maintaining the culture at 32°C. After transformation of 100ml of culture, the new D-serotype can be detected by immobilisation 12 hours after the temperature increase, by which time one fission has taken place (Fig. 75a). By 60 hours transformation is over and there are no cells showing the original G serotype.

The transformation of 1 litre of culture results in the more rapid appearance of the new serotype (Fig. 75b). This is not due to an increased fissions rate since both 100ml and 1 litre cultures divide at the same rate. The faster rate of transformation may be due to the greater heat-retaining capacity of large cultures. The higher temperature is probably more quickly established in the large culture and this may cause

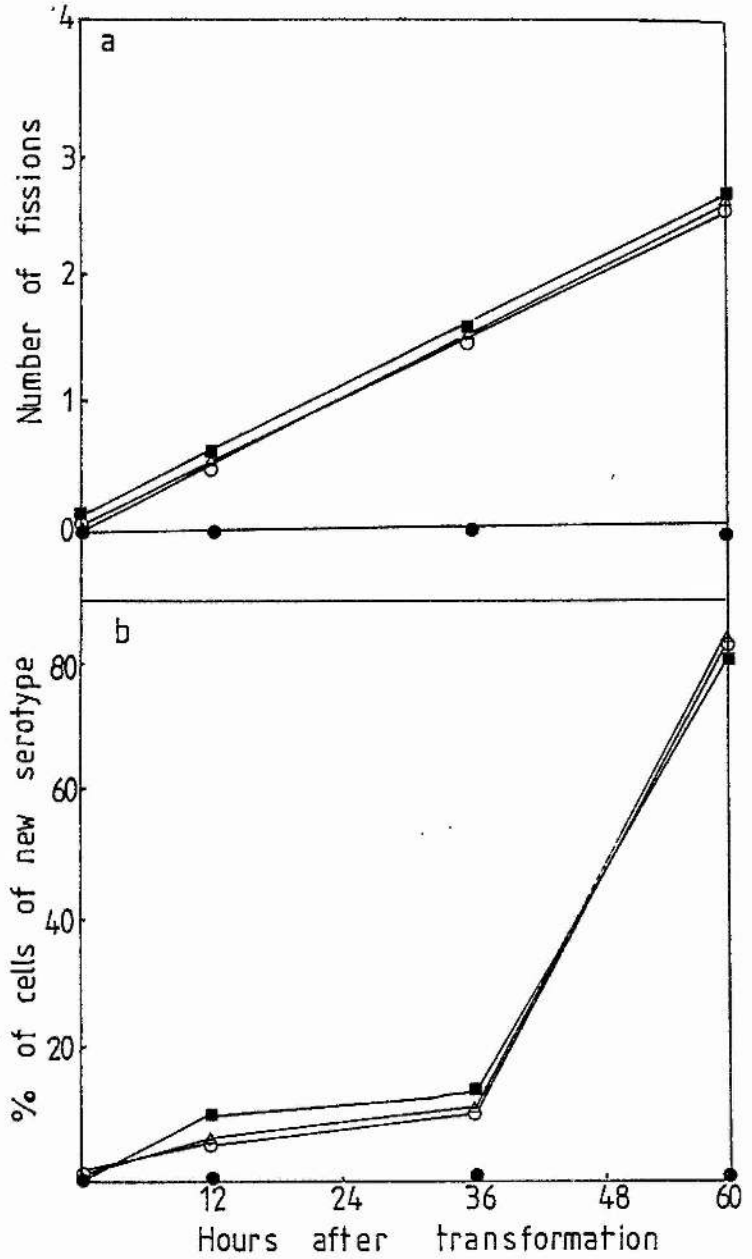


Fig. 76 a and b

The effect of metabolic inhibitors on a) fission rate and b) transformation

△ normal culture, ● + 20 µg/ml actinomycin D ○ + 20 µg/ml cordycepin
■ + 5 µg/ml ∝ - amanitin

a higher rate of transformation. It has been demonstrated by Beale (1957) that the rapidity of the temperature change influences the time for completion of serotype transformation.

(ii) The effect of metabolic inhibitors on transformation

Actinomycin D, if added at a concentration of 20µg/ml is sufficient to inhibit growth (Fig. 76a) also inhibits serotype transformation, as assayed by immobilisation (Fig. 76b). This agrees with the findings of Austin et al, (1967). It has, however, been demonstrated by Sommerville (1969) that, in the presence of actinomycin D, the new antigen can be detected by the method of autoradiography of precipitin arcs 16 hours after the transformation stimulus. It is possible that, in ciliates, actinomycin D does not act in the expected manner and is not totally inhibiting the synthesis of RNA under the conditions used.

The metabolic inhibitor cordycepin (3′-deoxyadenosine) is thought to inhibit mRNA synthesis by interfering with the process of 3′ polyadenylation (Penman et al., 1970), although the exact mode of action is uncertain (Maale et al., 1975). If transformation is mediated by a polyA$^+$ messenger RNA then the addition of 20µg/ml cordycepin to a culture should inhibit transformation. Fig. 76b shows that this is not the case. The transformation kinetics of a cordycepin-treated culture are indentical to those of an untreated culture, as are the growth kinetics (Fig. 76a). It is possible that, as with actinomycin D, this inhibitor does not affect ciliates in the same way as higher eucaryotes, or that the inhibitor is not incorporated by the paramecia.

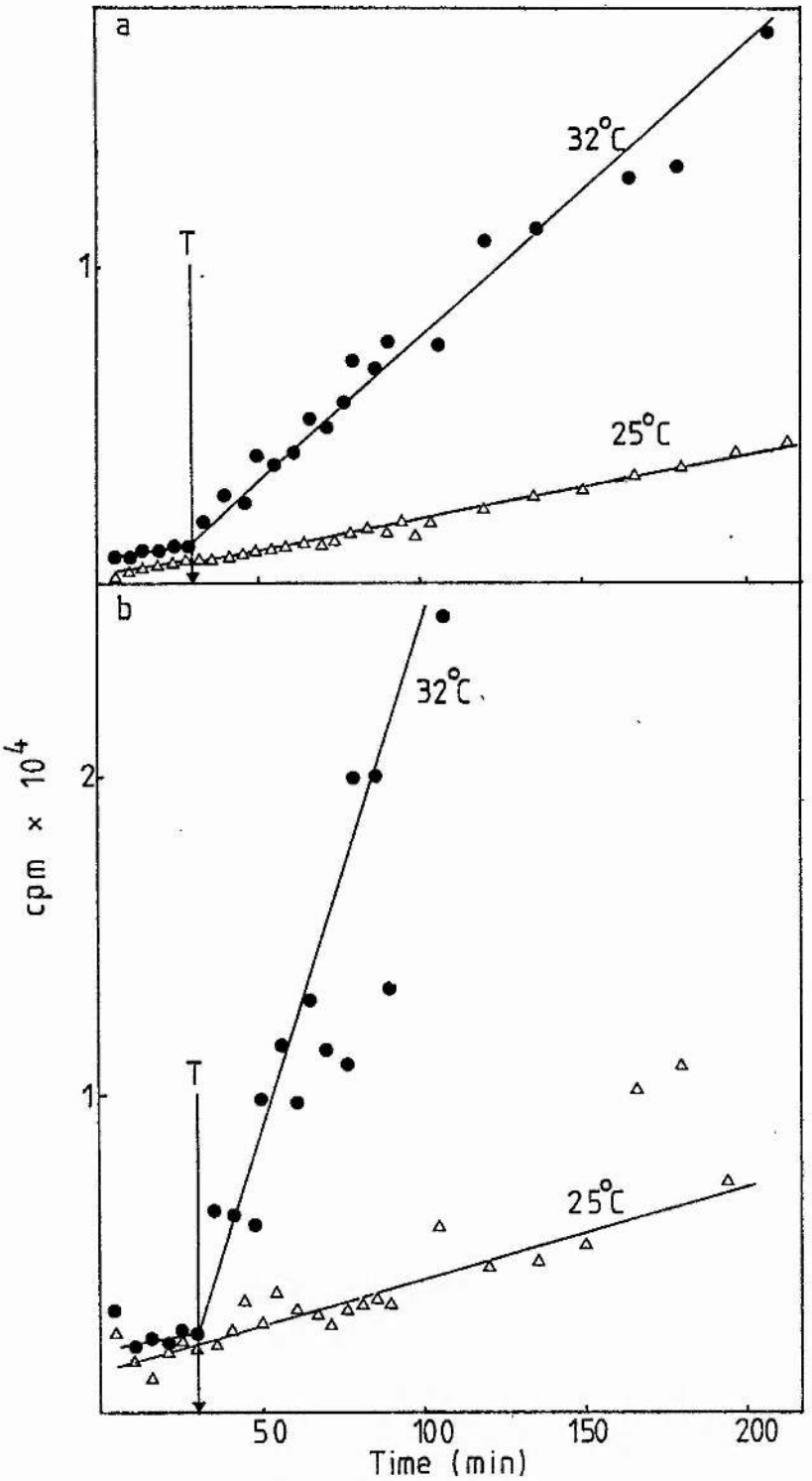


Fig. 77 a and b

The incorporation of a) ^{3}H-uridine and b) ^{14}C-amino-acid mixture into TCA precipitable material before and after the transformation of a culture of cells by an increase in temperature.

O - untransformed
● - transformed
T - time of temperature increase

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A similar result is obtained with 5ug/ml of α-amanitain (Fig. 76 a and b). Again it may be possible that 5μg/ml is too low to inhibit mRNA synthesis in paramecia or that the inhibitor may not enter the cell.

(iii) Changes in RNA and protein synthesis during transformation

Washed cells were labelled with ^{3}H-uridine or a ^{14}C-amino acid mixture as described in Chapter III. One sample of each was incubated at 25°C and a further sample, after 30 minutes of growth at 25°C, transformed by the addition of an equal volume of MS at 44°C. Fig. 77a shows the effect on RNA synthesis. The transformed culture, after transformation, incorporates ^{3}H-uridine into TCA precipitable material at a much higher rate than the non-transformed culture. The increased rate is established by the first sample point three minutes after transformation i.e. there does not appear to be any lag in the appearance of the higher synthetic rate. The rate of transformed cells is initially 5-6 times that of the untransformed culture but later falls to four times, this probably being due to the fact that the cells are labelled in MS. In these conditions the synthetic rate must be limited by the availability of endogenous metabolites, which would be more rapidly limiting at the higher rate.

Cells which had been growing for some time at 32°C were labelled with ^{3}H-uridine under the same conditions. These cells synthesised RNA at a rate similar to those which had just been transformed. Thus the higher rate of synthesis may be

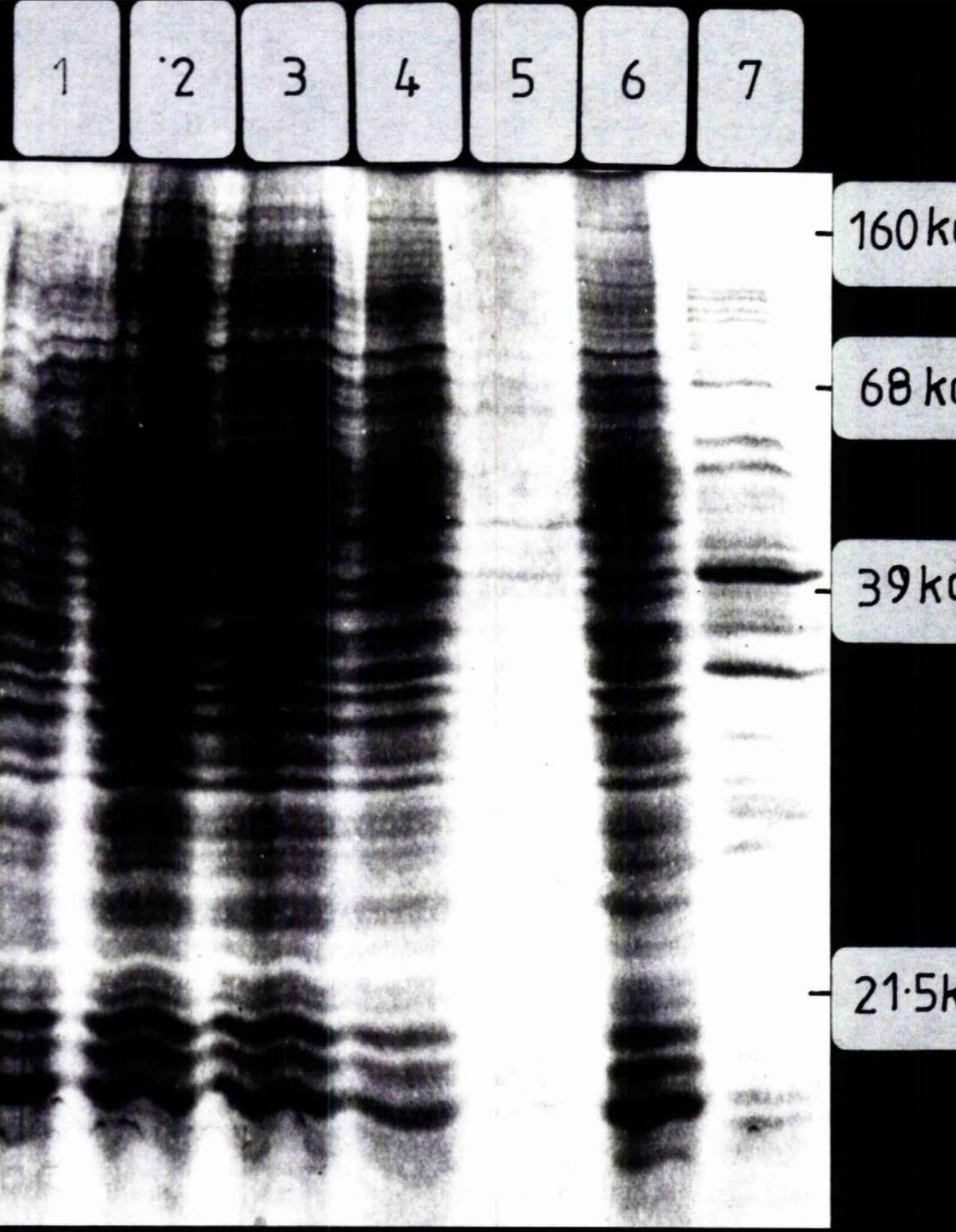

Fig. 78

Fig. 78

Acrylamide gel of protein samples from Paramecium at various times during transformation. For comparison, samples from G cells, D cells and Klebsiella aerogenes have also been analysed.

The molecular weights in kdaltons of marker proteins are indicated.

Track 1	G cells
Track 2	30 mins. after transformation
Track 3	3hr after transformation
Track 4	8hr after transformation
Track 5	24hr after transformation
Track 6	D cells
Track 7	Klebsiella aerogenes

entirely due to the higher temperature, although in most metabolic systems an increase of 7°C would not be expected to give rise to a rate increase of as much as five times.

A similar result is found for protein synthesis (Fig. 77b). Again there is an immediate increase in the synthesis rate, this time ten times the rate at 25°C, which is similar to the the rate of synthesis of cells established at 32°C. The immediacy of the effect of temperature in protein synthesis rate suggests that the integral increase in rate is due to an increase in the rate of translation of existing mRNAs, rather than to the translation of new mRNA sequences. In the latter case a lag before the rate increase might be expected.

There is, therefore, no evidence from this experiment of any special effect on RNA synthesis occuring at the time of transformation. However, as indicated in Chapter III, the method of labelling used may itself have some differential effect on cellular RNA synthesis. It is possible, therefore, that the normal pattern of RNA synthesis is not being monitored in this experiment.

(iv) Changes in cellular proteins during transformation

Changes in protein synthesis during transformation have been further examined by acrylamide gel electrophoresis of the proteins from samples of cells taken at various times during transformation (Fig. 78). There appear to be no major changes in the spectrum of proteins present as transformation proceeds and, as far as can be detected by this method, very little difference in the proteins of G and D cells. That these proteins

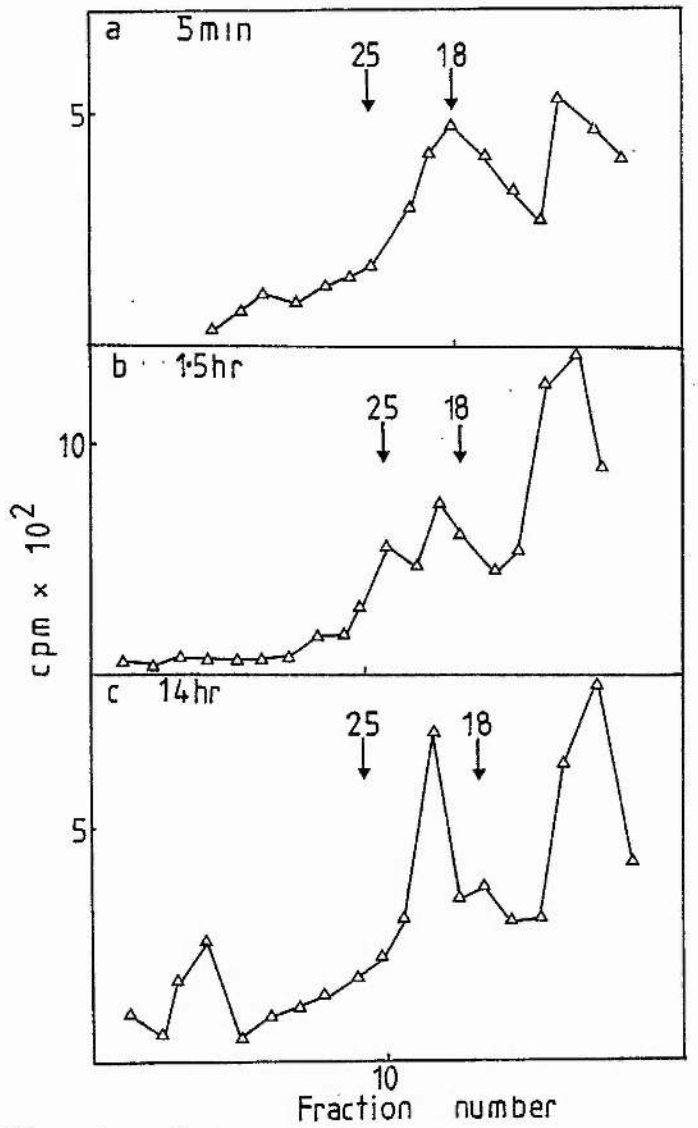

Fig. 79 a, b and c

The incorporation of ^{3}H-uridine into RNA after a 10 minute pulse at various times after transformation.

a) 5 minutes b) 1.5 hr. c) 14 hr.

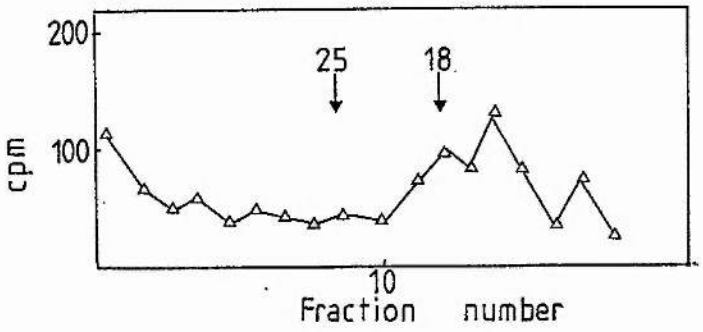

Fig. 80

Gradient analysis of polyA^{+}RNA isolated from cells labelled for 10 mins. with ^{3}H-uridine 14 hours after transformation

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originate from Paramecium rather than from bacteria is shown by track seven which is extracted protein from Klebsiella aerogenes and appears quite different from the protein spectrum of Paramecium. It is possible, of course, that there are differences in the amino acid sequence of some proteins but that these do not contribute to noticable changes in the molecular weight. For example, the antigen proteins of G and D cells are quite different in amino acid content and yet have very similar molecular weights (Jones, 1965., Steers, 1965).

(v) Changes in RNA synthesis during transformation

At various times during transformation, samples of cells were labelled for ten minutes with ^{3}H-uridine in MS containing sodium formate. As found before with this labelling method (see Chapter III), there is considerable labelling of the 20S peak (Fig. 79, a, b and c). This is most marked 14 hours after transformation. In addition there appears to be significant labelling at 14 hours of material which has a sedimentation value of 45S. This peak is generally somewhat variable in size and amount (see Chapter III) and may be due to molecular aggregation. PolyA^{+}RNA has been isolated from cells labelled at 14 hours (previous time points had given insufficient incorporation into polyA^{+}RNA). Here the pattern of labelling is quite different from that of total RNA (Fig. 80), the marked 20S peak being now totally absent. This is consistent with the results presented in Chapter III. The majority of rapidly labelled material at 14 hour ranges in size from 7-20S with a peak at 12S. This is the size expected of an

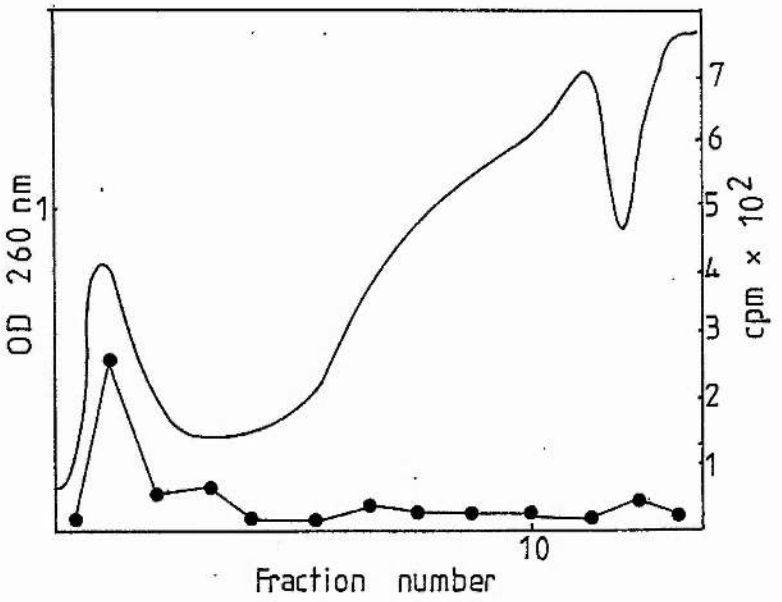

Fig. 81

Immunoprecipitation of fractions from a gradient of polysomes from cells labelled at 25°C with ^{35}S-labelled bacteria for 2hr. Anti-G serum has been used for the precipitation.

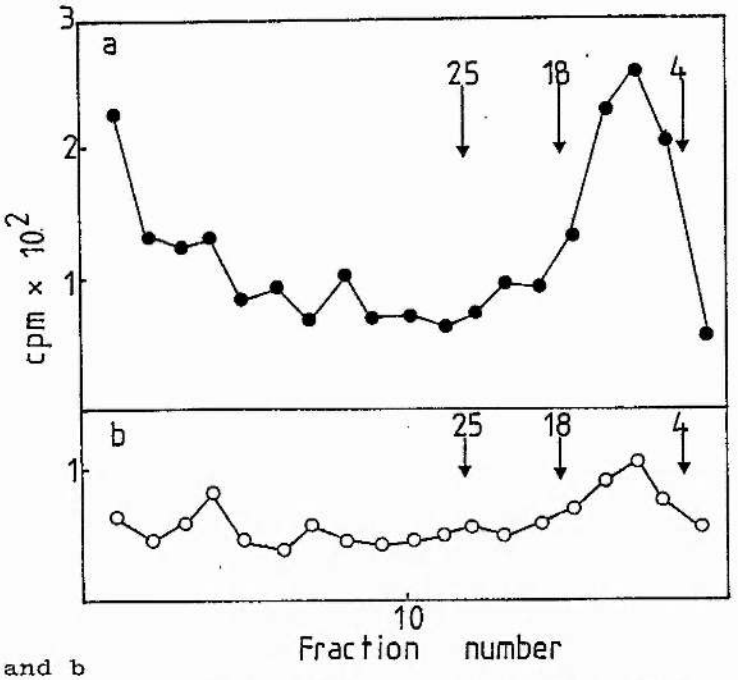

Fig. 82 a and b

Gradient analysis of RNA from polysomes of cells labelled for 30 minutes with ^{3}H-uridine at a) 25°C and b) 32°C under bacteria-free conditions.

In a) the polysomes have been precipitated with anti-G IgG and in b) with anti-D IgG

● cpm precipitated with anti-G
○ cpm precipitated with anti-D

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antigen mRNA coding for one subunit. A 12S peak is not prominent in the rapid labelling pattern of total RNA at any time after transformation and, if the 12S peak indeed corresponds to antigen mRNA, then this does not appear to be synthesised in large amounts during the process of transformation.

(vi) Detection of polysomes containing antigen mRNA

Polysomes containing antigen mRNA can be detected by the antibody reaction to their nascent polypeptides. The sizes of polysomes containing antigen mRNA have been detected in this way by Sinden (1973) who precipitated polysomes with ^{125}I-labelled antibody. Here cells were labelled with ^{35}S-labelled bacteria, the PMS prepared and polysomes separated by gradient centrifugation. Fractions were divided into two, one half treated with anti-G serum, the other half with non-immune rabbit serum. Fig. 81 shows that the only fractions giving significant amounts of specific precipitation are those of the membrane bound fraction and large (greater than 250S) polysomes. There is, in addition some specific precipitation near the top of the gradient. These results are in agreement with those of Sommerville (1967), although here ^{35}S-labelled bacteria have been used to label the cells rather than ^{14}C-leucine labelled bacteria.

(vii) Isolation of RNA from polysomes precipitated with antibody

Since it is possible to specifically precipitate polysomes with antiserum prepared against the antigen molecule, it should be possible to isolate the antigen mRNA. However, the conditions used to precipitate polysomes (two hour incubation at 37°C) would encourage endogenous ribonuclease activity and so were modified

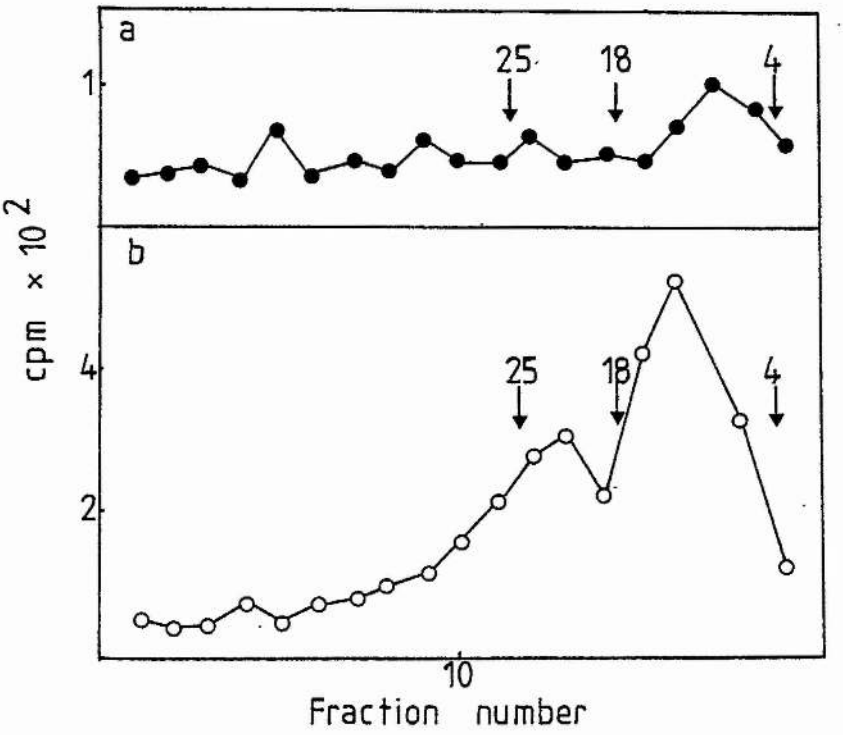

Fig. 83 a and b

Gradient analysis of RNA from polysomes of cells labelled with ^{3}H-uridine under bacteria-free conditions for 30 minutes.

In a) the RNA is from polysomes of cells grown at 25°C remaining un-precipitated with anti-G IgG

b) The RNA is from polysomes of cells grown at 32°C remaining un-precipitated with anti-D IgG.

● - cpm precipitated with anti- G

o - cpm precipitated with anti- D

for isolation of RNA. Firstly IgG was purified from the sera by passage through an ion-enchange column. Secondly the incubation was carried out at 0°C.

Cells were harvested labelled with ^{3}H-uridine for 30 minutes, the PMS prepared and incubated for one hour at 0°C with anti-G Ig G and for a further hour with an equal amount of anti-rabbit IgG. The antibody complex was pelleted and the RNA extracted. A similar experiment was carried out with labelled cells grown at 32°C incubated with anti-D IgG.

Labelled RNA precipitated by anti-G IgG appears to be mostly small, ranging in size from 4-18S (Fig. 82a). In addition there is some material which sediments at 36S and greater. Anti D precipitated RNA from cells grown at 32°C contains relatively little radioactivity (Fig. 82b) suggesting that these cells were not synthesising D antigen. This was later confirmed by the finding that these cells were not immobilised by anti-D serum.

Gradients of the RNA from the supernatant, i.e. from non-precipitated polysomes, show that, in the case of anti-G precipitation, the large 4-18S peak is much reduced as is, to a lesser extent, the large (>36S) peak (Fig. 83a). Fig. 83b shows the non-precipitated RNA from anti-D treated PMS. Here there is considerable labelling of the 12S peak and also of material sedimenting more rapidly than 22S.

A similar experiment was carried out with labelled G cells precipitated with anti-G IgG. Here, however, the RNA was heated to 70°C and centrifuged on a gradient under denaturing conditions to determine if any of the rapidly sedimenting RNA found in the previous experiment resulted from aggregation.

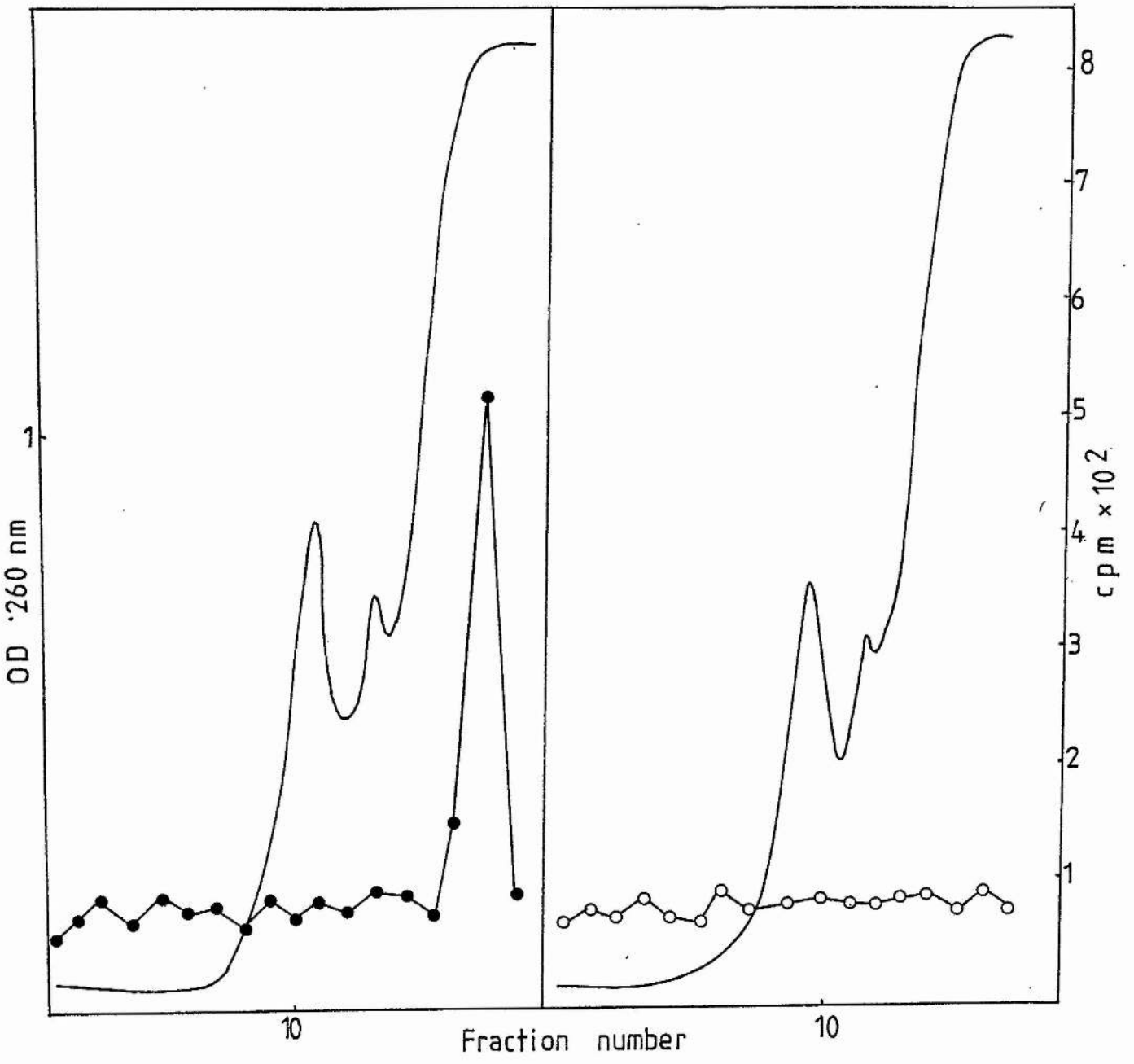

Fig. 84

Optical and radioactivity profile of RNA centrifuged under denaturing conditions after heating to 70°C for 5 minutes.

The RNA was isolated from polysomes of cells grown at 25°C and labelled for 30 minutes with ^{3}H-uridine under bacteria-free conditions. In a) polysomes were precipitated with anti-G IgG and in b) with anti-D IgG.

● - cpm precipitated with anti-G

○ - cpm precipitated with anti-D

However, the conditions used appear to encourage RNA breakdown and the labelled material now sedimented at less than 4S (Fig. 84).

Specific precipitation of polysomes with antibodies to the antigen molecule, appear to select RNA that sediments at 4-18S in size which may also form larger aggregates. Non-precipitated labelled RNA from the PMS also includes a large amount of 4-18S labelled material which probably represents the majority of mRNA molecules. There will, of course, be some non-specific precipitation in any precipitation reaction, but judging by Fig. 82b, this does not appear to be extensive, although it may contribute to the width of the peak in Fig. 82a. The antigen mRNA therefore appears to be approximately 10-12S in size, similar to the labelled polyA$^+$RNA peak in rapidly-labelled RNA from transforming cells. However the identity of this peak as antigen mRNA must await the *in vitro* synthesis of RNA and the identification of the labelled product by reaction to antiserum. As shown in Chapter III, polyA$^+$RNA from *Paramecium* can be translated in a cell-free protein synthesising system. One of the polypeptides synthesised had a molecular weight of 34kdaltons, about the size one would expect of an antigen subunit. Immunoprecipitation of the translated material however did not show precipitation of labelled material, although this could have been due to a low antiserum titre since added unlabelled antigen did not result in strong precipition arcs. It is interesting that the translation of *Paramecium* polyA$^+$RNA carried out by Hruby *et al.* (1977) shows very little synthesis of material in the 30-40kdaltons region.

4. Discussion

By rapidly increasing the temperature, cells of stock 168 of *Paramecium primaurelia* can be induced to transform from the G-serotype to the D-serotype. Cells expressing the new serotype can be detected by immobilisation within 12 hours of the temperature increase. However, by using more sensitive methods, the new antigen can be detected as early as 15 minutes after the temperature increase. (The sensitivity of various methods of detection are reviewed by Sommerville 1970). It seems likely that synthesis of the new antigen begins almost immediately after the transformation stimulus. The work of Balbinder and Preer (1955), although based on a different transformation system, also suggests that *de novo* synthesis of the new antigen begins very soon after the transformation stimulus.

In the transformation studied here, the transformation stimulus is a rise in temperature of 7°C. Such a temperature increase would be expected to give rise to an increase in the activity of a number of enzymes, so it is difficult to distinguish the effects of the temperature increase *per se* from the effect of a transformation stimulus on the activity of a number of genes. It has been suggested by Pasternak (1967) that there is an inhibition of macronuclear synthesis in the early period following transformation induced by antiserum, although there appeared to be an increase in the rate of RNA synthesis in patulin-induced transformation.

With temperature induced transformation, the increase in the rate of RNA (and protein) synthesis can be attributed to the effect of temperature on metabolic rate, rather than to any special effect on differential genetic activity since cells established at 32°C also have a similar higher rate of RNA and protein synthesis compared to those grown at 25°C. However, as discussed in Chapter III, the method of labelling may be biased towards mitochondrial RNA synthesis and obscuring any changes in macronuclear RNA synthesis.

An examination of the proteins present at various times during transformation, indicates that there is very little, if any, change in the protein spectrum during transformation, or indeed between G and D cells. It is possible however that the pattern of proteins being synthesised varies throughout transformation. Such changes would be detectable in an autorad of labelled cells but such proteins might not contribute to the bulk of proteins present in the cells.

The data on the complexities of polyA$^+$RNA from G and D cells indicates considerable differences in the spectrum of polyA$^+$RNA present (see Chapter III). Such differences have also been suggested by the work of Gibson (1970) who finds variations in the sequences of RNA molecules throughout transformation. Variations in RNA synthesis have been examined here by labelling cells for ten minutes at various times during transformation and examining the species of RNA which become labelled. As previously described in Chapter III a 20S peak is prominent in the labelling pattern. In other respects also, the labelling pattern is similar to non-transforming cells suggesting that there may be little change in the

pattern of RNA synthesis other than in rate. An examination of the transcription units of cells at various stages of transformation shows no difference in the morphology or frequency of the two types of transcription unit (see Chapter III).

PolyA$^+$RNA from transforming cells (at 14 hours) is also similar to that of non-transforming cells and in both a 12S peak is prominent. Although this is the molecular weight expected of the antigen mRNA if it codes for one subunit only, the antigen mRNA can only be tentatively assigned to the 12S peak until it can be demonstrated that this fraction can be transcribe *in vitro* into antigen protein. The antigen mRNA, after all, may not be polyadenylated.

In an attempt to isolated the antigen mRNA, polysomes have been precipitated with antiserum prepared against the antigen molecule. It has previously been demonstrated by both Sommerville (1967) and Sinden (1973) that polysomes can be precipitated by virtue of their nascent polypeptides. This has been repeated here and, in agreement with earlier work, the fractions which appear to contain antigen-bearing polysomes are those of the membrane-bound and large polyribosomes.

In order to investigate the RNA associated with such polysomes, cells were labelled with ^{3}H-uridine, the polysomes precipitated with antiserum and the RNA extracted. This RNA was compared to RNA from polysomes which had remained unprecipitated with antiserum.

There appeared to be an enrichment of material in the 4-18S region which had a peak at 10-12S. There also appeared

to be specific precipitation of larger material which had peaks at 36 and 49S. This larger material could be due to aggregation but an attempt to exclude such effects was hampered by the sensitivity of the RNA to denaturing conditions. Most of the non-precipitated material also contained RNA sedimenting at 4-18S, and it is possible that there in some non-specific precipitation in the antiserum precipitated RNA. However there does appear to be some enhancement of the material precipitated in this size-range and it is probable that the antigen mRNA is included in this size class. This would agree with the work of Sinden (1973) who found the smallest polysomes which could be precipitated with antiserm were of sufficient size to code for a 35,000 dalton polypeptide. Such polysomes would contain an mRNA which would sediment at approximately 12S.

The effect of the metabolic inhibitors cordycepin and α-amanitin on transformation is surprising. Neither inhibit transformation, unlike actinomycin D and chloramphenicol which both inhibit transformation if used at high concentration. Neither cordycepin nor α- amanitin, used at 5 μg/ml, had any effect on the fission rate. This can be explained in a number of ways. The inhibitor may not enter the cell, but this seems unlikely since the feeding activity of the paramecia must inevitably include some of the culture fluid, although the inhibitors may of course be destroyed in the food vacuole. However, actinomycin D and chloramphenicol both appear to be taken up by the cell and so it is more likely that the ineffectiveness of cordycepin and α- amanitin is due to the

insensitivity of the polymerases of Paramecium to the concentrations used. The use of higher concentrations might elucidate this point.

In molecular terms, the transformation process does not appear to be a traumatic event, there appearing to be very little change in RNA and protein synthesis other than that which one might expect from a temperature increase. Temperature changes must be a common phenomenon in the natural environment of Paramecium. However, Paramecium does transform under the influence of many unnatural factors, e.g. antiserum and various chemicals. Such transformations may be fundamentally different in terms of molecular events, and may only appear to be similar by virtue of a change in the expression of one particular gene out of, possibly, many genes. Comparisons between transformation processes stimulated by different agents may be misleading since they may influence antigen gene expression by different mechanisms. This must be borne in mind when considering possible forms of control of the expression of the antigen genes. This aspect will be discussed more fully in the general discussion and possible mechanisms of control of the antigen-genes considered in the light of the molecular biology of Paramecium .

5. Summary

RNA and protein synthesis have been examined during the course of temperature induced serotype transformation in Paramecium. There appears to be very little change either in the pattern of RNA synthesised or of the spectrum of proteins present during transformation. Although there is an increase

in the rate of both RNA and protein synthesis after transformation, this can be attributed to the increased temperature rather than to any special effect of transformation.

An attempt to isolate the antigen mRNA has suggested that it may be approximately 12S in size, in agreement with the observations of Sinden (1973) on the size of antiserum-precipitable polysomes.

Neither of the metabolic inhibitors cordycepin or ∝-amanitin influenced transformation, although both actinomycin D and chloramphenicol inhibit transformation if used at high concentrations. It is possible that Paramecium is insensitive to cordycepin and ∝-amanitin.

GENERAL DISCUSSION

The serotype transformation system of protozoa has been studied at length by a number of authors (reviewed by Beale, 1957., Preer, 1968., Sommerville, 1970., Finger, 1974). Although serotype transformation seems to involve a simple gene switch which can be stimulated by a number of factors, very little is known about the control of this process. The transformation system, although interesting in its own right, should provide an ideal system for the study of genetic control mechanisms in lower eucaryotes. The use of the ciliate Paramecium to study this system is historical in origin. Although the related ciliate Tetrahymena is generally a more convenient experimental organism and the flagellate Trypanasoma is, in terms of human welfare, economically more important, the serotype system has been studied in Paramecium since it appears to be more ameniable to genetic and biochemical analysis that the related systems of these other ogranisms. A considerable body of data now exists on the genetics of the serotype transformation system and on the synthesis of the antigen molecules in Paramecium (see Sommerville, 1970., Finger, 1974).

Paramecium is a typical ciliate and therefore is amongst the simplest of the eucaryotes. The relevance of studies on the ciliates to higher organisms can, of course, be questioned. One might ask if the ciliates, as simple eucaryotes, possess eucaryotic features possibly in a primitive form, or whether they have evolved some special features of their own which bear little relation to higher eucaryotes. With regard to genetic

control mechanisms, the occurrence of the two quite different nuclei which, in the hypotrichs at least, show considerable genetic differences (Ammermann et al., 1974., Lauth et al., 1976) is a uniquely ciliate feature and may indicate some major differences in this respect.

In the work described in this thesis, the macronuclear DNA of stock 168 of P. primaurelia has been studied with respect to both genome organisation and transcription and a comparison has been made with higher eucaryotes. In both respects, there are many features similar to those of higher eucaryotes. However there are some significant differences, and any possible mechanism of gene control must take into account the special characteristics of the ciliate genome.

The first important aspect is the possession by the ciliates of two types of nuclei. This, as previously discussed, has implications for the control of gene expression. In the hypotrichous group of ciliates, for example, there are considerable changes in the informational content of the genome during development of the macronucleus from the micronucleus (Ammermann et al., 1974., Lauth et al., 1976). This may, of course, not be a general feature of macronuclear development in the ciliates (e.g. Yao and Gorovsky, 1974) and in Paramecium it has not been rigorously demonstrated that the informational content of the two nuclei is different, due to the difficulty in purifying micronuclear DNA. An indirect assessment, however, suggests that there may be little sequence loss during development (see Chapter II). However, any analysis of the type carried out here and by other authors (Allen and Gibson, 1972., Soldo and Godoy, 1972., Cummings, 1975), based on a comparison

of sequence complexity, cannot rule out minor, but possibly highly significant, changes in the DNA organisation. A more specific analysis of the DNA, possibly with regard to a particular genetic sequence, might elucidate this point. Some changes which could concievably occur during macronuclear development are "loss" or "gain" of certain sequences. The highly polyploid nature of the macronucleus could provide a mechanism for such changes by under-replication or over-replication of certain sequences. Another possible change would be the removal of the intervening sequences which are found in the genes of most eucaryotes examined (reviewed by Abelson, 1979). Although an intervening sequence within a protein coding gene has not been demonstrated in a ciliate, there is an intervening sequence in the 25S ribosomal DNA of Tetrahymena (Wild and Gall, 1979., Zang and Cech, 1980).

Whether or not there are any changes during the development of the ciliate macronucleus, the fully developed macronuclear genome is quite simple (see Table 1, Chapter II). In Paramecium there seems to be very little repetitive DNA, a maximum of 2% being detected in the more rapidly reassociating fraction. It is difficult to measure accurately such a small percentage of the genome and this value may be anything from 0-5% . Cummings (1975) has shown that the macronuclear genome of Paramecium contains repeated 25S RNA genes and tRNA genes accounting for 0.16% and 0.28% of the genome respectively. The small amounts of repetitive DNA detected in Chapter II may contain other genes which, like the ribosomal and tRNA genes, are found to be repetitive in most organisms studied,

for exaple the 5S genes and histone genes which in Oxytricha macronuclear DNA appear to be repeated, (Elsevier et al., 1978). Although Paramecium does not lack repetitive DNA it clearly does not possess the much larger amounts found in higher eucaryotes. This is a feature which must be taken into account in considering control models based on the presence of large amounts of repetitive DNA.

The macronuclear genome of Paramecium has a sequence complexity of 1.6×10^8 nucleotides. This is sufficient to code for approximately 8×10^5 "average sized genes". In all eucaryotes studied to date, the genome complexity appears not to be fully "expressed" as transcribed sequences and generally only a small proportion of the genome is transcribed. In an organism with a relatively simple genome such as Paramecium (20 times that of E. coli) one might expect a higher proportion to be transcribed. In the slime mould Dictyostelium which has a sequence complexity similar to that of Paramecium (Firtel and Bonner, 1972) 56% of the genome can be transcribed although rather less is transcribed under any one set of growth conditions (Firtel, 1972). In other lower organisms the percentage of genome transcription is somewhat variable being only 4% in Achyla (Timberlake et al., 1977) and higher in yeast at 20% (Hereford and Rosbash, 1977). Work by Christianson (1970) suggests that 3-4% of the genome is transcribed in Tetrahymena. This seems a somewhat low value and in Paramecium the value is higher, ranging from 5-22% depending on the method used to estimate the value and the

conditions of growth. The exact value for the percentage of the genome which is transcribed is difficult to determine accurately. By the two methods employed, saturation of DNA sequences with excess polyA$^+$RNA and cDNA polyA$^+$RNA hybridisation kinetics, it appears that cells grown at 32^{o}C transcribe more DNA into polyA$^+$RNA than those grown at 25^{o}C, although a number of unknown factors could account for this effect.

However, even the maximum estimate of 22% is not particularly high. While it may be possible that polyA$^-$RNA accounts for a significant fraction of the genome complexity, this seems unlikely since the data of Gibson (1970) suggests a saturation value of 10% for rapidly labelled RNA which would include polyA$^-$RNA as well as polyA$^+$RNA. Another possibility is that cells grown under different conditions transcribe different RNA sequences. This appears to be the case for cells grown at 25^{o}C and 32^{o}C and, although the difference amounts to only a few percent, it is possible that other additional sequences are transcribed under different growth conditions.

The value for the percentage genome transcription derives largely from the least frequently represented RNAs. However, like the RNA of other organisms, the polyA$^+$RNA of Paramecium falls into a broad range of intracellular concentrations which, in an organism having equally represented DNA copies, implies that some genes are transcribed at a higher frequency, or have more stable RNA products, than others.

An interesting feature of the least frequent class of

RNA, is the calculation of the number per cell of molecules of each species, which ranges from 1-4 x 10^3. In most cells the least frequent RNA species are present only 1-10 times per cell (see Lewin, 1975). The higher value in Paramecium may indicate that all of the 840 copies of the expressed genes are being transcribed.

An examination of the hybridisation of polyA$^+$RNA to heterologous cDNA shows that, as indicated by the saturation of total DNA with polyA$^+$RNA, there are differences in the sequences of polyA$^+$RNA present in cells grown at 25°C and 32°C. Such differences exist for RNA species which cover the whole spectrum of intracellular frequencies.

It has proved difficult to investigate these differences between the two cell states in terms of RNA synthesis. Paramecium feeds on bacteria and does not grow well in axenic medium (see Chapter I). The method of feeding has several implications for the investigation of RNA synthesis. Firstly, paramecia must contain many degradative enzymes which will include nucleases. Although DNA can be isolated from Paramecium, particularly from purified macronuclei, the variation in yield and the general presence of short lengths of DNA indicate nuclease activity. A more serious probelm for experimental analysis is the presence of large amounts of endogenous ribonuclease activity which is extremely difficult to inhibit. Any methods which involve prior separation of sub-cellular components, for example the polysome-containing fraction and which therefore could not be used with the more potent protein denaturing ribonuclease inhibitors, was inadequate for the

preparation of RNA (see Chapter III). The alternative method of homogenising intact cells in a solution containing such ribonuclease inhibitors, produced higher molecular weight RNA and an improved yield of polyA$^+$RNA. However, this method was inadequate for short term radioisotope labelling of RNA since bacterial RNA is inevitably present. Consequently, a non-bacterial method of labelling was used. This, however, produced a labelling pattern unlike that of other cells, with predominant 20 and 18S peaks. This labelling was insensitive to the metabolic inhibitors actinomycin D and α-amanitin and, although it has not been demonstrated that these inhibitors act in Paramecium as they do in higher eucaryotes, it seems probable that the labelling of 20 and 18S peaks is due neither to polymerase I nor II activity. It is more likely that this labelling is mitochondrial in origin, the 20S peak corresponding to the large mitochondrial rRNA and the 18S peak possibly being due largely to the smaller mitochondrial rRNA which in Tetrahymena is 14S in size (Chi and Suyama, 1970) but which in Paramecium may concievably be larger.

In view of the extensive labelling of mitochondrial RNA and because of the generally low levels of incorporation into RNA, it has proved difficult to measure the size range of rapidly labelled RNA. However it can be said that there is little labelled RNA which sediments in excess of 30S. This observation does not of course exclude the possibility that the labelling of higher molecular weight RNA is inhibited under the labelling conditions employed, or that it is synthesised

but has a very short half life. The latter possibility may well be the case since an electron microscopic examination of primary RNP transcripts shows them to be much longer than one would expect if RNA is transcribed only to its final polyA$^+$RNA length. Furthermore, processing of the rRNA precursor in Tetrahymena is much more rapid than processing of the rRNA precursor of higher eucaryotes (Prescott et al., 1971). This rapidity of processing may well extend to processing of primary transcripts to their polyA$^+$RNA length.

Whether or not this is the case, it is clear that, in Tetrahymena and probably in Paramecium also, rapidly-labelled RNA is not much bigger than polyA$^+$RNA suggesting the absence of an hnRNA stage in RNA synthesis. This appears to be true also of other lower eucaryotes and in general, rapidly-labelled RNA in the same size as, or not much bigger than, polyA$^+$RNA or messenger RNA. (Braun et al., 1966., Prescott et al., 1971., Firtel and Lodish, 1973).

The labelling pattern of paramecia during transformation was examined in the hope of identifying the mRNA for the surface antigen molecule which, like the protein itself, should be synthesised in large amounts. The synthesis of a major RNA species was not observed, although perhaps an alternative labelling method might give more significant results. An attempt to isolate antigen mRNA from polysomes showed that a 12S species of RNA could be purified from antiserum-precipitated polysomes, This is the expected sedimentation value of a mRNA large enough to code for one subunit of the antigen molecule and is in agreement with the immunoprecipitation

data of Sinden (1973). The identification of the 12S peak as the antigen mRNA, however, awaits its translation in an in vitro protein synthesis system.

Total polyA$^+$RNA isolated from cells grown at 25°C has been translated successfully in a wheatgerm cell-free system. Repeated attempts to translate Paramecium polyA$^+$RNA in a cell-free rabbit reticulocyte system have been unsuccessful, possibly due either to the sensitivity of the reticulocyte preparation to some factor in the polyA$^+$RNA or to the lack of some component required for adequate translation of ciliate mRNA.

In the wheatgerm translation a 34,000 dalton polypeptide was observed. This in the approximate size of an antigen subunit molecule. However, the translated proteins did not react with antiserum prepared against the antigen protein, although this could have been due to the low titre of the antiserum (see Chapter I).

Clearly much work remains to be carried out on the serotype transformation system particularly with regard to the isolation of antigen mRNA. However, in the light of both this work and that of other authors, possible mechanisms for the control of the antigen genes can be considered.

Many of the earlier models for the control of the expression of the antigen genes were influenced by the elegant control mechanisms studied in bacteria (see Lewin, 1974). It has been suggested, for example, that the antigen acts as an inducer of its own synthesis by inactivating a repressor molecule which is produced from a closely linked regulator

gene (Finger, 1967). This has been prompted by the finding that the serotype of an unstable clone can be stabilised by the presence of a particular antigen, (Finger, 1967).

The observation that antiserum to the expressed surface antigen can induce transformation (Beale, 1948., Dryl, 1959) supports the conclusion that the antigen is in some way involved in the control of its own synthesis but neither of these observations would suggest that this is necessarily at the level of gene transcription.

The idea of a closely linked regulatory sequenced has some support. Each serotype allele has a charcteristic environmental range over which it is stably expressed. The stability charcteristics are inherited with the serotype (Beale, 1957) suggesting that stability is a property of the antigen molecule itself or is due to some closely linked gene or sequence. Further evidence for the existence of regulatory sequences associated with the antigen gene comes from studies on stock 192 of P. primaurelia which show that cells homozygous at the G-locus are incapable of expressing the G-serotype, but can express it in a heterozygous strain. This suggests that control sequences for the G locus are absent from stock 192 but can be supplied by the presence of another allele.

In high eucaryotes sequences which are closely linked to the histone gene and which appear to have a regulatory role have been described (Grosschedl and Birnstiel, 1980). One sequence which may be related to the bacterial promotor sequence (Gannon et al., 1979) appears to regulate the site

of initiation of transcription while another sequence appears to control the rate of transcription. The existence of sequences of the latter type was suggested by Guille and Quetier (1973) who proposed that sequences concerned with quantitative control of gene expression would be closely linked to structural genes. It is conceivable that the stability of a particular surface antigen could be controlled by the level of synthesis which may, in turn, be influenced by upstream sequences of the type found by Grosschedl and Birnstiel (1980).

The observations on Paramecium while suggesting existence of regulatory elements, are not accounted for by a mechanism such as the classic Jacob-Monod system. The serotype transformation system appears to be considerably more complex than the inducible enzyme systems of bacteria from a number of points of view. Not only are up to 11 alternative loci excluded at any one time, but the control mechanism also discriminates between the alleles at a given locus (Finger and Heller, 1964) and possibly even between cistronic regions within the locus (Finger et al., 1966). The sensitivity of antigen gene expression to a wide variety of environmental factors, and the co-ordinate expression of all the elements involved in serotype expression, suggests a complex control mechanism.

This complexity, relative to bacteria is not unexpected in view of the greater structural and genetic complexity of Paramecium. Being eucaryotes they have, in any case,

the added complications of histones (. Isaaks and Santos, 1973) and a nuclear membrane.

It may not be possible to operate a Jacob-Monod type control mechanism in an organism much more complex than a bacterium. Indeed, it has been suggested by Scherrer and Marcaud (1968) that a large number of genes can be more easily controlled by a multistep process than by the direct selection of single genes. This theory of "cascade regulation", which involves the control of gene expression at a number of different levels, has gained increasing support from examination of RNA synthesis in higher eucaryotes.

While there must be control of gene expression in higher eucaryotes at the level of transcription, there is considerable evidence for post-transcriptional control. This can act at a number of levels, but the most obvious candidate for control mechanisms is the processing of RNA which may involve a large number of steps, each potentially regulated (reviewed by Darnell, 1973., 1979). Although a considerable amount is known about the structure of the precursor and product mRNAs of several genetic sequences (see Lewin, 1975), little is known about the actual mechanism of processing. However it is clear that, in Paramecium, such a potential control of gene expression must be either very rapid or absent altogether since no hnRNA can be detected, and it seems possible that the control of gene expression in Paramecium may largely be at the level of transcription, although translational control cannot of course be excluded.

A number of models have been suggested for the control of transcription in higher eucaryotes, most including some

function for repetitive DNA (Britten and Davidson, 1969., Georgiev, 1969., Becker, 1972., Paul 1972, Guille and Quetier, 1973). Much of the repetitive DNA of higher eucaryotes is in the form of short related sequences which are interspersed between longer unique DNA. For this reason the families of repetitive DNA have been assigned a control function, a repetitive family controlling the co-ordinate expression of adjacent structural genes at a number of chromosomal sites (e.g. Britten and Davidson, 1969, Davidson et al., 1977).

In Paramecium co-ordinate gene expression must be involved in several processes, for instance in organelle differentiation and the development of the macronucleus. Even the synthesis of the antigen molecule very probably involves the co-ordinate expression of different sub-unit genes. It has been suggested by Nanney (1963) that the antigen genes have large regions of related sequence and that the mRNA of an antigen gene can either bind to the corresponding DNA or to related DNA either stimulating or inhibiting further transcription, respectively. This would suggest that either the antigens have a very similar amino-acid sequence which appears not to be the case or that the coding region is proceeded by a non-translated but transcribed region which is similar in all the antigens. This would require at least some repetition of the DNA. However, there appears to be very little repetitive DNA in the macronucleus of Paramecium and as such, a general model of the control of gene transcription relying on families of repeated sequence is not appropriate to Paramecium although the sort of model proposed by Nanney involving an extremely limited degree of

repetition cannot be excluded by the renaturation data.

One of the most complex phenomena associated with serotype transformation is that of the cytoplasmic state (Beale, 1957). This is essentially the sum of extranuclear components and is determined by a number of factors which include the genetic background, the environmental conditions and the history of the cytoplasm e.g. the time since conjugation or recent starvation. The cytoplasmic state can be altered by changing the environmental conditions and it is suggested that it is the change in cytoplasmic state which causes the change in the expression of the antigen genes. The factors in the cytoplasmic state which influence antigen gene expression are not known but may include RNA sequences. As indicated in Chapter III, the different cytoplasmic states of cells expressing the G serotype and D serotype have many differences in the sequences of RNAs being transcribed. It is possible that a change in environmental conditions brings about a major change of gene expression, possibly the co-ordinate expression of a new set of genes but which probably includes some transcribed by the previous cytoplasmic state. This is similar in essence to the batteries of genes proposed by Britten and Davidson (1969).

Such a change may not have any major effect on the proteins present since cellular functions could be carried out by similar proteins coded for by different genes. For

example the antigens are transcribed from different genes and have different amino acid contents, yet they have a similar molecule size and appear to perform a similar function (Jones, 1965., Steers, 1965., Reisner et al., 1969).

It has not been demonstrated that a particular antigen is more efficient in the conditions under which it is expressed than another. A change in serotype expression may have no functional significance and could merely represent one of the possibly many changes in gene expression which accompany a change in cytoplasmic state. The reason for such a change in gene expression in response to environmental conditions is not evident, but may relate to the situation in early embroys where there is considerable transcription and turn-over of RNA which does not appear to be immediately translated (c.f. Davidson, 1976).

The control of the antigen genes, in molecular terms, may be very simple, but the situation is complicated by the extensive additional changes in gene expression which accompany serotype transformation. It would be interesting to examine other serotypes, particularly those induced by some factor other than temperature to find if there are further changes in the expression of the genome under these different conditions. As far as control of the antigen gene itself, it is clearly desirable to purify the mRNA and to prepare a cDNA copy. With the cDNA one could examine directly the transcription of the antigen gene and possibly discover what factors affect its trans-

cription. But it seems likely that, even in such a simple eucaryote as Paramecium that the control of gene expression is considerably more complex than in bacteria.

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